Aeromonas salmonicida - Epidemiology, whole genome sequencing, detection and in vivo imaging

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Aeromonas salmonicida

Epidemiology, whole genome sequencing, detection and *in vivo* imaging

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List of manuscripts

This thesis includes the following original manuscripts that have either been submitted, accepted or are published online.

Manuscript I (accepted in Journal of Fish Diseases)

Title: Infection routes of *Aeromonas salmonicida* in rainbow trout monitored *in vivo* by real-time bioluminescence imaging

Authors: Simona Bartkova, Branko Kokotovic, Inger Dalsgaard

Manuscript II (published online in Journal of Fish Diseases)

Title: Detection and quantification of *Aeromonas salmonicida* in fish tissue by real-time PCR

Authors: Simona Bartkova, Branko Kokotovic, Helle Frank Skall, Niels Lorenzen, Inger Dalsgaard

Manuscript III (submitted to Frontiers in Microbiology)

Title: Epidemiology and genetics of *Aeromonas salmonicida* using whole genome sequencing

Authors: Simona Bartkova, Pimlapas Leekitcharoenphon, Frank Møller Aarestrup, Inger Dalsgaard

List of Abbreviations

AFLP	Amplified fragment length polymorphism
A-layer	Virulence associated surface protein array
ARGs	Antibiotic resistance genes
A. salmonicida	*Aeromonas salmonicida* subsp. *salmonicida*
BHI	Brain heart infusion
BLI	Bioluminescence imaging
bp	Base pairs
β	Beta
CBB	Coomassie brilliant blue
CC	Clonal complex
CFU	Colony-forming units
ECPs	Extracellular products
ELISA	Enzyme-linked immunosorbent assay
$FMNH_2$	Reduced riboflavin phosphate
GCAT	Glycerophospholipid:cholesterol acyltransferase
GFP	Green fluorescence protein
IROMPs	Iron-regulated outer membrane proteins
LB	Luria Bertani
LPS	Lipopolysaccharide
LUX	Light upon eXtension
M-CGH	Microarray-based comparative genomic hybridization
MLST	Multilocus sequence typing
MLST-v	MLST with housekeeping genes and virulence associated genes
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Randomly amplified DNA polymorphism fingerprinting analysis
Real-time PCR	Quantitative real-time polymerase chain reaction
R plasmids	Plasmids carrying antibiotic resistance genes
SIF	Stress-inducible furunculosis
SLVs	Single-locus variants
SNP	Single nucleotide polymorphism
ST	Sequence type
TSA	Tryptic soy agar
T2SS	Type II secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
VBNC	Viable but non-culturable cells
VIB	Veal infusion broth
WGS	Whole genome sequencing

Summary

Aeromonas salmonicida subsp. *salmonicida* is a bacterial fish pathogen that is the causative agent of furunculosis, a septicemic infection responsible for great losses in aquaculture around the world. In Denmark furunculosis was first seen in freshwater in the 1950s, though currently the infection causes problems in sea reared rainbow trout (*Oncorhynchus mykiss*) production. Outbreaks occur repeatedly during stressful conditions such as elevated temperatures, in spite of commercial vaccines being applied. Besides seemingly lacking adequate protection, the vaccines also produce undesirable side effects. Antibiotics are therefore used as treatment, which due to the possibility of developing resistance is neither a favorable nor sustainable solution. To complicate things further, it is possible that fish can be carriers of *A. salmonicida* and transfer the bacterium from freshwater to the sea where they develop septicemia when exposed to stressful sea-rearing conditions and high temperatures. By use of traditional bacteriological methods, continuous investigation of bacterial diagnostics on samples from different rainbow trout farms in Denmark was done, while studying the following three aspects of the concerns regarding *A. salmonicida*.

First, we focused on investigation of the route of entry and initial dissemination of *A. salmonicida* in fish. This was done by tracing the bacterium using *in vivo* bioluminescence imaging. A Danish strain was transformed with a plasmid vector containing a green fluorescence protein gene and bacterial luciferase genes that served as fluorescent and bioluminescent reporters respectively. The transformed *A. salmonicida* was used in a series of immersion experiments where fish were followed over a 24-hour period. Results showed that probable main colonization sites of *A. salmonicida* were the gills and the dorsal and pectoral fins. This was followed by dissemination through internal organs. Although optimization and further immersion experiments are needed, our results indicated that this tool could be a valuable approach for visualizing *A. salmonicida* in fish.

Focus was subsequently turned to finding a sensitive method for detecting *A. salmonicida* in infected and possible carrier fish. For this, a previously developed quantitative real-time polymerase chain reaction (real-time PCR) targeting the *aopP* gene located on *A. salmonicida* plasmid pAsal1 was assessed. The real-time PCR and bacterial culturing were employed for preliminary screening of *A. salmonicida* in 40 fish from Danish fresh- and seawater farms. *A. salmonicida* was detected by real-time PCR in freshwater farm fish showing no sign of disease, indicating possible presence of carrier fish. Out of five examined organs: spleen, kidney, intestine, gills and brain in each fish, *A. salmonicida* was most frequently detected in the spleen, brain and intestine, indicating that these three organs could play an important role in *A. salmonicida* infection. The real-time PCR exhibited highly sensitive detection of *A. salmonicida* as well as a high reproducibility and efficiency, though due to the fact that not all *A. salmonicida* seem to possess the target plasmid pAsal1, another sensitive detection method with a different and/or complementary target would need to be employed to be certain of avoiding false negatives.

The final focal point of this thesis revolved around obtaining knowledge on genetic and virulence variation as well as epidemiology of the disease causing Danish *A. salmonicida*. Due to high homogeneity among the *A. salmonicida* subspecies population, standard molecular methods for

bacterial typing cannot distinguish among *A. salmonicida* isolates. Whole genome sequencing was therefore applied on 99 Danish *A. salmonicida* isolated between years 1980 and 2014 from different geographical regions, one Scottish strain and the type strain NCIMB 1102. Sequences of the *A. salmonicida* were *de novo* assembled and then examined for presence of plasmids, virulence and iron acquisition proteins, and antibiotic resistance genes. The chromosome was also examined for single nucleotide polymorphisms that were aligned and subjected to Bayesian temporal tree reconstruction using the published genome of *A. salmonicida* A449 as reference. Main results revealed that there have been four major introductions of *A. salmonicida* into Denmark, *A. salmonicida* are highly homogenous with the exception of certain plasmids and virulence factors encoded on these plasmids, and nine *A. salmonicida* harbored several worldwide known genes encoding resistance against antibiotics. This study provided valuable information regarding the Danish disease causing *A. salmonicida*.

Sammendrag (summary in Danish)

Aeromonas salmonicida subsp. *salmonicida* er en fiskepatogen bakterie, som forårsager sygdommen furunkulose, der medfører store tab i akvakultur over hele verden. I Danmark blev furunkulose første gang beskrevet i ferskvand i 1950'erne, men i øjeblikket forårsager sygdommen problemer i regnbueørred (*Oncorhynchus mykiss*) produceret i havbrug. Udbrud forekommer gentagne gange under stressende forhold, såsom forhøjede vandtemperaturer, på trods af at kommercielle vacciner anvendes. Udover tilsyneladende at mangle tilstrækkelig beskyttelse, medfører vaccinerne også uønskede bivirkninger. Antibiotika anvendes derfor som behandling, men på grund af muligheden for udvikling af resistens er dette hverken en positiv eller holdbar løsning. For at komplicere tingene yderligere, kan fisk være såkaldte skjulte bærere af *A. salmonicida* og overføre bakterien fra ferskvand til havet, hvor sygdom udvikles når fiskene udsættes for stressende forhold. Ved brug af traditionelle bakteriologiske metoder, blev der udført kontinuerlig undersøgelse på fisk fra forskellige dam- og havbrug i Danmark, samtidig blev det forsøgt at klarlægge nogle af de ovennævnte problemer vedrørende *A. salmonicida*.

Først har vi fokuseret på at undersøge, hvordan *A. salmonicida* kommer ind i fiskene, samt bakteriens indledende udbredelse inde i fiskene. Dette blev gjort ved at spore bakterien med *in vivo* bioluminescens imaging. En dansk *A. salmonicida* stamme blev transformeret med en plasmid vektor, der indeholder et gen kodende for et grønt fluorescerende protein og bakterielle luciferase gener kodende for bioluminescence. Den transformerede *A. salmonicida* blev brugt i et foreløbigt bad eksperiment, hvor fiskene blev fulgt over en 24-timers periode. Resultaterne viste, at sandsynlige fasthæftnings steder af *A. salmonicida* var gællerne og ryg- og brystfinner. Dette blev efterfulgt af udbredelse til de indre organer. Selvom der er behov for optimering og yderligere bad eksperimenter, viste foreløbige resultater, at denne metode kunne være et værdifuldt redskab til at visualisere *A. salmonicida* i fisk.

Fokus blev derefter vendt imod at finde en følsom metode til påvisning af *A. salmonicida* i inficerede og mulige bærerfisk. Til dette blev en tidligere udviklet kvantitativ real-time polymerasekædereaktion (real-time PCR) rettet mod *aopP* genet, der er lokaliseret på *A. salmonicida* plasmidet pAsal1 vurderet. Real-time PCR og dyrkning af bakterien blev brugt til en foreløbig undersøgelse af *A. salmonicida* i 40 fisk fra danske dam- og havbrug. *A. salmonicida* blev påvist med real-time PCR i dambrugs fisk uden tegn på sygdom, hvilket tyder på tilstedeværelse af bærerfisk. Følgende fem organer: milt, nyre, tarm, gæller og hjerne fra hver fisk blev undersøgt, hvorfra *A. salmonicida* hyppigst blev påvist i milten, hjernen og tarmen, hvilket viser at disse tre organer kan spille en vigtig rolle i *A. salmonicida* infektionen. Real-time PCR udviste høj følsomhed for påvisning af *A. salmonicida* samt en høj reproducerbarhed og effektivitet, men på grund af at ikke alle *A. salmonicida* har plasmidet pAsal1, er det nødvendigt at anvende en anden følsom påvisnings metode rettet imod et andet gen, for at være sikker på at undgå falske negative resultater.

Det sidste omdrejningspunkt for denne afhandling drejer sig om at skaffe viden om den genetiske variation, samt oprindelse og spredning af danske *A. salmonicida* som forårsager sygdom. På grund af høj homogenitet i *A. salmonicida* bakterierne kan standard molekylære metoder til bakteriel

typning ikke skelne mellem forskellige *A. salmonicida* isolater. Helgenomsekventering blev derfor anvendt på 99 danske *A. salmonicida* stammer der blev isoleret i perioden 1980 til 2014 fra forskellige geografiske områder. Yderligere blev en skotsk bakterie og type bakterien NCIMB 1102 undersøgt. Sekvenser af bakterierne blev *de novo* samlet og derefter undersøgt for tilstedeværelse af plasmider, virulens og jern protein sekvenser og gener for antibiotika resistens. Kromosomet blev også undersøgt for nukleotid polymorfenheder (SNPs), der blev brugt til konstruktion af et Bayesian fylogenetisk træ ved hjælp af det publicerede genom af *A. salmonicida* A449 som reference. De vigtigste resultater viste, at der har været fire store introduktioner af *A. salmonicida* i Danmark, at *A. salmonicida* er meget homogene med undtagelse af visse plasmider og virulensfaktorer kodet på disse plasmider, og at ni *A. salmonicida* havde flere globalt kendte gener, der koder for antibiotika resistens. Studiet resulterede i værdifuld viden om *A. salmonicida* bakterier, der forårsager sygdom i akvakultur i Danmark.

Introduction

Development in aquaculture

According to the Food and Agriculture Organization of the United Nations (2016), aquaculture is the fastest growing food-producing sector. This intensified fish-farming combined with elevated water temperature due to global warming, creates epidemiological opportunities for pathogens and thereby causing problems in aquaculture (Tan et al., 2002; Ganusov and Antia, 2003; De Silva and Soto, 2009; Pulkkinen et al., 2010). One of the problems is that the fish immune system becomes adversely affected by physiological stress arising during high stocking density and abnormal climate conditions such as prolonged rise in temperature (Harvell et al., 1999; Vargas-Chacoffa et al., 2014). The above mentioned conditions also create a favorable environment for activity of pathogens that otherwise would typically remain dormant (Lafferty, 2009) and general increase of virulence and/or transmission rate of pathogens (Marcogliese, 2008; Marcos-López et al., 2010).

In contrast to the rest of the world's aquaculture industry, the Danish industry has in the recent years remained stagnant. However, fish consumption in Denmark has increased during the last few years (Miljø- og Fødevareministeriet, 2015), leading to the Danish authorities' proposal for expanding aquaculture in order to increase production (Miljø- og Fødevareministeriet, 2014). Use of antibiotics in aquaculture has decreased due to implementation of vaccines. However, in comparison with other animal productions in Denmark, marine aquaculture lies at the top in use of antibiotics along with pig production when calculated in DAPD (Defined animal daily dose per 1,000 animals per day), which includes changes in live biomass and thus enables comparison of different animals (DANMAP, 2012). An increase in production and a possible rise in water temperature will not alleviate this problem. On the contrary, it will increase the potential threat of highly virulent pathogens emerging.

Problem of furunculosis

Furunculosis is a septicemic infection caused by the highly homogenous Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* (Bernoth et al., 1997; Garcia et al., 2000). Though *A. salmonicida* can be present in fish without inducing signs of disease where the infection is said to be in a 'covert' stage (Hiney et al., 1997). It is believed that these carrier fish transfer the bacterium from freshwater farms out to seawater farms, where the fish develop septicemia when exposed to stressful sea-rearing conditions and high temperatures (Dalsgaard and Madsen, 2000; Pedersen *et al.*, 2008). *A. salmonicida* can be diagnosed via traditional bacteriological methods (Dalsgaard et al., 1994; Austin and Austin, 2007), however, detection of the bacterium in carrier fish based on these methods has thus far been unsuccessful (Dalsgaard and Madsen, 2000). New and more sensitive methods need to be developed in order to detect *A. salmonicida* in carrier fish.

Furunculosis was first described from freshwater farms in Denmark in the 1950s (Rasmussen, 1964) and now causes great problems in seawater rainbow trout farms. There is only one vaccine against *A. salmonicida* that is licensed for Danish rainbow trout, which is the Norwegian commercial vaccine

AlphaJect 3000, initially developed for Atlantic salmon (*Salmo salar*) against vibriosis and furunculosis (Pharmaq, 2016). Although the vaccine is being implemented, outbreaks of furunculosis still occur (Dalsgaard and Madsen, 2000; Pedersen *et al.*, 2008), causing substantial economic losses in aquaculture. Side effects, likely caused by oil adjuvants, have also been reported (Haugarvoll et al., 2010; Mutoloki et al., 2010). In order to develop an effective strategy for preventing furunculosis, a more effective vaccine against Danish *A. salmonicida* needs to be developed and knowledge about the epidemiology, genetic and virulence variation of the Danish disease causing *A. salmonicida* isolates needs to be obtained.

Objective of this thesis

The objective of this PhD project was to contribute to ongoing research on resolving the current concerns of furunculosis in Danish rainbow trout production by: 1) investigating route of entry and dissemination of *A. salmonicida* in fish in order to study the host-pathogen relationship that could provide new knowledge for improvement of detection and sampling strategies of the bacterium, 2) developing a highly sensitive method for detection of *A. salmonicida* in possible carriers and fish showing signs of disease, and 3) determining the epidemiology, genetic and virulence variability of the Danish *A. salmonicida* isolates that could aid in the development of an effective strategy for preventing furunculosis.

Chapter 1: *Aeromonas salmonicida* subsp. *salmonicida*

1.1 Background and taxonomy

Aeromonas salmonicida subsp. *salmonicida* is an important bacterial fish pathogen, which was originally isolated at a German freshwater farm by Emmerich and Weibel (1894) and was given the name *Bacterium salmonicida*. Subsequently it was proposed by Griffin et al. (1953) to place the bacterium in the genus *Aeromonas* and re-classify the name of the species as *Aeromonas salmonicida* (Snieszko, 1957). The genus of *Aeromonas* has also gone through many taxonomic re-classifications and was eventually placed in the family *Aeromonadaceae* by Colwell et al. (1986). Although the species of *Aeromonas salmonicida* was first thought to be homogenous, by use of biochemical and molecular methods it has thus far been divided into five subspecies: *salmonicida, masoucida, achromogenes, smithia, and pectinolytica* (Austin, 1993; Wiklund and Dalsgaard, 1998; Kozinska et al., 2002; Beaz-Hidalgo et al., 2008; Studer et al., 2013). The four latter subspecies all belong to the so called "atypical" group, while subspecies *salmonicida* is the only *Aeromonas salmonicida* known as "typical" and is the causative agent of furunculosis. Subspecies *salmonicida* is also the focal point of this PhD project and is in this thesis referred to as *A. salmonicida*.

1.2 Biochemical and morphological characteristics

The bacterium *A. salmonicida* is Gram-negative, facultative anaerobic, non-motile, psychrophilic and consists of coccoide cells with the measurement of 0.5-6.0 x 1-2 µm (Marsh, 1902; Griffin et al., 1953; Cipriano and Austin, 2011). One of the most basic characteristics of the subspecies is that colonies produce a brown water-soluble pigment after growth on agar in the presence of 0.1% tyrosine or phenylalanine for two to four days (Fig. 1) (Marsh, 1902; Griffin et al., 1953; Boone et al., 2001; Cipriano and Austin, 2011). The subspecies must, however, not be identified solely based on this characteristic, since some *A. salmonicida* strains do not produce this pigment (Wiklund et al., 1993; Koppang et al., 2000) while some other bacteria like *A. hydrophila* also produce diffusible brown pigment (Austin and Austin, 2012). There are also numerous well-known biochemical characteristics, which are frequently used for identification. This includes production of catalase and cytochrome oxidase and gelatin liquefaction, although exceptions have been found (Böhm et al., 1986; Wichardt et al., 1989; Chapman et al., 1991). Generally the bacterium has also been reported of being positive for the following carbohydrates and glycosides: glycerol, glucose, fructose, galactose, mannitol, mannose, maltose, dextrin, glycogen, starch, aesculin and salicin, as well as being positive in L-arabinose but negative in D-arabinose and not being able to convert tryptophan into indole (Dalsgaard et al., 1994). Though, gas production by *A. salmonicida* from fermented glucose might be weak in some strains or as seen with a strain from Canada, gas might not be produced (Dalsgaard et al., 1994). *A. salmonicida* negative for acid production from mannitol and hydrolysis in aesculin have, however, also been found (Austin et al., 1989).

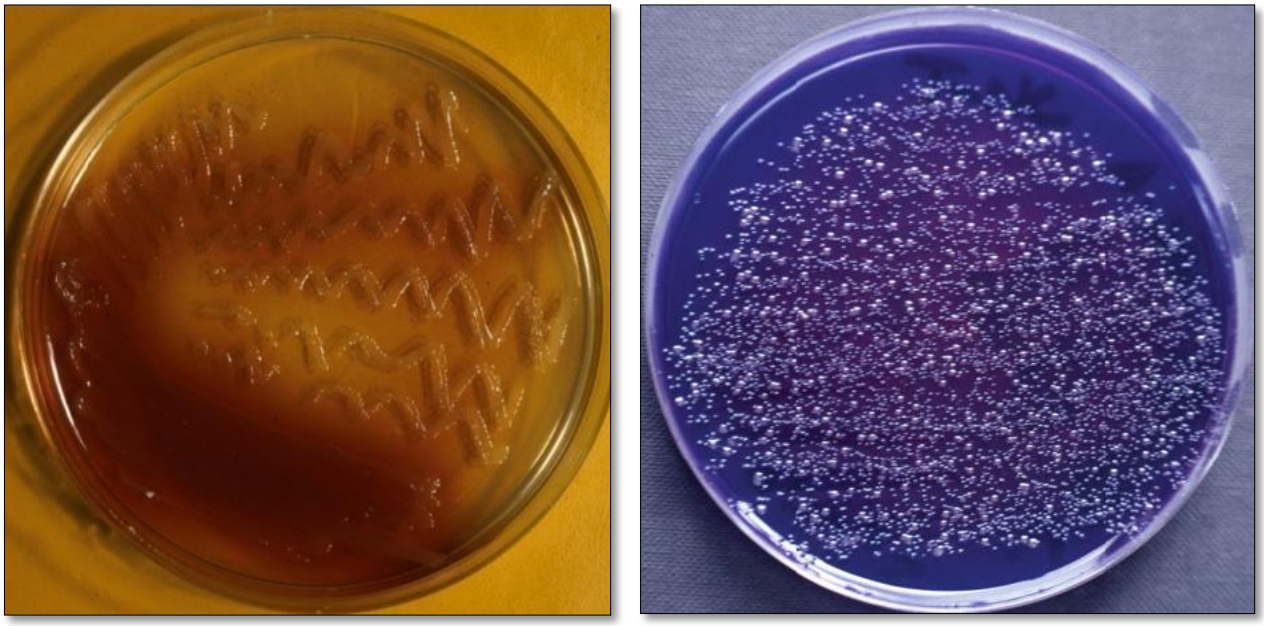

Figure 1. *A. salmonicida* colonies grown on tryptic soy agar (left) and on Coomassie brilliant blue agar (right, from Austin and Austin, 2012)

After incubation on nutrient agar for about 24 hours at 18 - 20^{o}C, *A. salmonicida* colonies are flat and punctiform with a diameter of less than 1 mm (Griffin et al., 1953; Boone et al., 2001). It is not until three to four days of growth (Griffin et al., 1953; Boone et al., 2001) that the colonies become circular, convex and entire with a diameter of 1 - 2 mm, which is the characteristic morphology of *A. salmonicida* colonies on solid media. Colonies also become friable at this point, such that if pushed, they can slide on the agar surface without being damaged (Duff and Stewart, 1933; Munro and Hastings, 1993; Boone et al., 2001). When the bacterium is grown on blood agar for 2 - 4 days the colonies also become grayish in color and form a zone of haemolysis around them due to production of β-haemolysis (Griffin et al., 1953; Dalsgaard et al., 1994).

Additionally, *A. salmonicida* has the ability to autoagglutinate in static liquid media and form "smooth" and "rough" colonies on solid media (Arkwright, 1912; Williamson, 1928; Udey, 1978; Kay and Trust, 1997). These characteristics are generally based on the presence or absence of the virulence associated surface protein array called the A-layer, with A-layer positive *A. salmonicida* strains autoagglutinating and forming "rough" colonies (Johnson et al., 1985; Dalsgaard et al., 1994; Kay and Trust, 1997; Austin and Austin, 2012). A-layer negative strains that autoagglutinate have, however, also been found (Johnson et al., 1985) and single bacterial isolates can form morphologically different colonies on solid media (Anderson, 1972; Dalsgaard et al., 1994; Austin and Austin, 2012). This is supported by our findings, where all except one of 101 sequenced *A. salmonicida* possessed the A-layer protein sequence (Manuscript III), though only 76 of the isolates seemed to autoagglutinate in static liquid media (unpublished results).

Another property of *A. salmonicida* that has been used in order to distinguish A-layer positive from A-layer negative strains is the ability to bind the dyes Coomassie brilliant blue (CBB) (Wilson and Horne, 1986; Cipriano and Bertolini, 1988; Dalsgaard et al., 1994; Austin and Austin, 2012) and

Congo red (Ishiguro et al., 1985; Dalsgaard et al., 1994). The A-layer positive *A. salmonicida* bind the dye and grow as dark blue and red colonies on tryptic soy agar (TSA) with added CBB and Congo red respectively, while the A-layer negative colonies grow as whitish colonies. Markwardt et al. (1989) also reported that CBB agar could be used to distinguish A-layer positive *A. salmonicida* from other bacteria in mixed populations isolated from clinical samples. However, Teska and Cipriano (1993) reported that bacteria such as *Aeromonas hydrophila*, *Pasteurella multocida* and various *Pseudomonas* species can also grow with dark blue colonies on CBB agar and dark blue colonies must therefore be subcultivated and tested for other characteristics before any confirmations can be made regarding their identification.

1.3 Cultivation

The recommended medium for isolation and cultivation of *A. salmonicida* by diagnostic manuals has usually been brain heart infusion (BHI) agar or TSA and both have been used in several studies (e.g. Beaz-Hidalgo et al., 2008b; Beaz-Hidalgo et al., 2013; Austin and Austin, 2012). TSA has the advantage of the possibility of supplementation with CBB or Congo red for selection of A-layer positive *A. salmonicida* as mentioned in the previous section, however, selection using this medium is not consistent and Austin and Austin (2012) reported that *A. salmonicida* "rough" colonies that are associated with A-layer and virulence are recovered better on BHI agar than TSA. *A. salmonicida* can also grow on common laboratory media such as Luria Bertani (LB) and blood agar and the latter has especially been used frequently (e.g. Böhm et al., 1986; Dalsgaard et al., 1994; Pedersen et al., 2008) due to the usual *A. salmonicida* production of β-haemolysis on this medium. The choice of liquid medium for cultivation of *A. salmonicida*, which enables the possibility for observing autoagglutination of A-layer positive isolates, has also varied between studies e.g. veal infusion (VIB) broth (Dalsgaard et al., 1994), Trypticase soy broth (Ishiguro et al., 1981) and BHI broth (Johnson et al., 1985). All *A. salmonicida* used in the present thesis were always cultivated in VIB broth and isolated on blood agar, with the exception of one study (Manuscript I) where LB agar with added ampicillin and BHI broth were also employed due to special circumstances.

Ideal growth conditions for *A. salmonicida* include aerobic conditions and pH from 5.3 to 9.0, although this can vary depending on the composition of the culture medium (Griffin et al., 1953). For many years the optimal growth temperature for *A. salmonicida* has been reported as being 22 - 25°C (Griffin et al., 1953; Brenner et al., 2005) and the maximum growth temperature 34.5°C (Griffin et al., 1953). However, it has now been reported by several studies that when *A. salmonicida* is grown at 25^{o}C, or in some studies even 22^{o}C, some of the plasmid encoded virulence genes can become inactivated or lost due to plasmid rearrangement or loss of the plasmids (Ishiguro et al., 1981; Stuber et al., 2003; Daher et al., 2011). This has led to Daher et al. (2011) suggesting that *A. salmonicida* should be grown at a maximal temperature of 20^{o}C. All *A. salmonicida* used in this thesis were grown at 20^{o}C.

1.4 Virulence

There are many virulence factors that *A. salmonicida* possesses, which can be used against the defense mechanisms of the host in order to establish an infection (Reith et al., 2008; Beaz-Hidalgo

and Figueras, 2013; Dallaire-Dufresne et al., 2014). Though, not all virulence factors are equally important and some are even non-functional (Reith et al., 2008). In fact, the virulence mechanism of *A. salmonicida* has proven to be a complex and intertwined system (Reith et al., 2008; Beaz-Hidalgo and Figueras, 2013; Dallaire-Dufresne et al., 2014).

One of the known virulence components of *A. salmonicida* that are important for attachment of host cells and entry into the host are adhesins e.g. the surface layer and pili (Austin and Austin, 2007; Reith et al., 2008). Though, it is only the A-layer, a tetragonal protein array that is associated with lipopolysaccharides (LPSs) on the cell surface, which has been described as being a major virulence factor of *A. salmonicida* (Udey and Fryer, 1978; Ishiguro et al., 1981; Phipps et al., 1983; Trust et al., 1983; Chart et al., 1984). This protein, encoded by the *vapA* gene (Chart et al., 1984), also displayed high sequence homogeneity in 101 sequenced *A. salmonicida*, of which 99 isolates were from Denmark and only one Danish isolate did not harbor the protein (Manuscript III). The A-layer also has a high proportion of hydrophobic amino acids; a property that increases hydrophobicity of the bacterial surface (Phipps et al., 1983). In agreement, Dalsgaard et al. (1994) reported that the "rough" colony forming strains of *A. salmonicida* belived to be A-layer positive had a more hydrophobic outer cell surface than the strains forming "smooth" colonies. When studying *in vitro* cultured macrophages Trust et al. (1983) found that A-layer positive *A. salmonicida* had an enhanced ability to associate with the macrophages, which was also enabled by the increase in hydrophobicity. The increased hydrophobicity is also responsible for A-layer positive *A. salmonicida* strains' ability to autoagglutinate and to adhere to host tissue, while the A-layer negative strains have been reported to be avirulent (Udey and Fryer, 1978). The association of hydrophobicity with aggregation and sedimentation is also supported by Enger and Thorsen (1992), who added that the property could also play a role in the ecology of the bacterium outside its host, since *A. salmonicida* was detected by use of immunofluorescence in sediment beneath net pens, seawater, and surface film from samples at fish farms with furunculosis outbreaks. Nevertheless, A-layer negative virulent as well as A-layer positive non-virulent *A. salmonicida* have been observed, exemplifying that *in vitro* tests do not necessarily give an accurate result when assessing virulence of *A. salmonicida* (Johnson et al., 1985; Ellis, 1997), which as suggested by Olivier (1990) should be assessed through *in vivo* challenges instead.

Other virulence factors that have been reported as being important for virulence of *A. salmonicida* are extracellular products (ECPs) such as haemolysins, aerolysins, lipopolysaccharides, proteases and various toxins, which have been the subject of many *A. salmonicida* studies over the years (Ellis et al., 1981; Ellis et al., 1988; Ellis, 1997; Austin and Austin, 2007; Beaz-Hidalgo and Figueras, 2013). Already in 1953, Griffin et al. believed that the observed β-haemolysis and gelatin liquefaction on blood and gelatin plates respectively could be related to the characteristic tissue lesions of furunculosis and thus caused by production of protease enzymes. Years later in a study by Ellis et al. (1981), it was possible to reproduce the furunculosis associated lesions when ECPs were injected into rainbow trout. In another study, Ellis et al. (1988) found that protease and haemolysin activity promoted the development of lesions in fish, though there was another yet uncharacterized factor of the ECPs that was lethal for the fish. One ECP that has been proven to be lethal for fish is the toxin glycerophospholipid:cholesterol acyltransferase (GCAT) (Lee and Ellis, 1990). The GCAT protein sequence was present in all 101 sequenced *A. salmonicida* and displayed similar sequence

homogeneity as the A-layer (Manuscript III). This toxin can also aggregate with LPSs, creating a GCAT/LPS complex which is even more toxic than GCAT by itself (Lee and Ellis, 1990). Surprisingly, when the encoding genes of either GCAT or another reportedly important toxin (serine protease AspA) were mutated, virulence of the *A. salmonicida* mutant was not altered (Vipond et al., 1998).

Iron-regulated outer membrane proteins (IROMPs) are responsible for uptake of siderophore-iron complexes and heme (Hirst et al., 1994; Najimi et al., 2008, 2009). They are thus believed to play a significant role in virulence of *A. salmonicida*, since iron is an essential nutrient and acquisition of iron is necessary for survival within the host and maybe also the aquatic environments (Hirst et al., 1994; Najimi et al., 2008, 2009; Reith et al., 2008; Ebanks et al., 2013). Iron acquisition by *A. salmonicida* can be siderophore dependent, by which the siderophores remove iron from proteins of the host and enable entrance of the iron into the bacterial cell through IROMPs (Najimi et al., 2008; 2009). Though, iron can also be obtained by a siderophore independent system that functions as a way to remove iron from host hemoglobin (Ebanks et al., 2004; Najimi et al., 2008; 2009). With regard to *A. salmonicida*, IROMPs have also shown to have an inhibitory effect on host transferrin thereby improving the bacterial resistance against the host's phagocytes (Magnadottir, 2010). Investigation of genes involved in siderophore biosynthesis and IROMPs indicated that both systems seem to be conserved among the homogenous *A. salmonicida* (Fernandez et al., 1998; Najimi et al., 2009), as supported by the findings of Manuscript III, while other iron mechanism proteins sequences are not present in all *A. salmonicida* (Najimi et al., 2009).

Secretion systems have also been known for their significance for virulence and three have been described in *A. salmonicida*, which includes type II (T2SS), III (T3SS), and VI (T6SS) (Reith et al., 2008). T2SS is in charge of enzyme degradation and toxin secretion, while T6SS enables injection of effector proteins into the cytoplasm of the host cells (Reith et al., 2008). Much like T6SS, the main function of T3SS is also injection of effector proteins and toxins into the cytosol of host cells, but has also other functions including prevention of phagocytosis by leukocytes and establishing a systemic infection in the host (Burr et al., 2003; Stuber et al., 2003; Burr et al., 2005; Mota and Cornelis, 2005; Dacanay et al., 2006; Ebanks et al., 2006; Rasch et al., 2007; Fast et al., 2009; Dallaire-Dufresne et al., 2014). Interestingly, both T3SS and T6SS associated genes are moreover situated in the chromosome as well as plasmids (Reith et al., 2008; Fehr et al., 2006). Though, notably T3SS is the only virulence factor proven to be essential for virulence and toxicity of *A. salmonicida*, since inactivation of the T3SS structural proteins in *A. salmonicida* has always rendered the *A. salmonicida* mutants non-virulent in both *in vitro* and *in vivo* studies (Burr et al., 2002; Burr et al., 2003; Stuber et al., 2003; Burr et al., 2005; Dacanay et al., 2006; Froquet et al., 2007; Fast et al., 2009). Thus far there have been described five T3SS effector proteins in *A. salmonicida*: AexT (an ADP-ribosylating toxin encoded on the chromosome), AopH (a tyrosine phosphatase encoded on the plasmid pAsa5 along with its chaperone SycH), AopO (a serine/threonine kinase encoded on the plasmid pAsa5 along with its chaperone SycO), Ati2 (an inositol polyphosphate 5-phosphatase encoded on the plasmid pAsa5 along with its chaperone Ati1) and AopP (this toxin is involved in inhibition of IkB protein kinase activation and is encoded on plasmid pAsal1) (Dacanay et al., 2006; Fehr et al., 2006; Reith et al., 2008; Dallaire-Dufresne et al., 2013). Both the effector proteins and structural proteins of T3SS are thought to play a role in *A. salmonicida* survival within the host cell, though when

compared only the structural proteins proved to be vital for virulence (Dacanay et al., 2006; Fast et al., 2009). An additional factor supporting the important implication of T3SS in virulence is that the secretion system is widely spread among pathogenic Gram-negative bacteria, such as other *Aeromonas* spp., *Yersinia* spp., *Salmonella* spp., and *Pseudomonas* spp., where it has also been confirmed to be vital for virulence of the pathogens (Chacón et al., 2004; Vilches et al., 2004; Mota and Cornelis, 2005; Vilches et al., 2009). Interestingly, both certain T3SS effector protein and structural protein sequences e.g. the highly studied AscV, were absent in 24% of the 101 sequenced *A. salmonicida*; all of which were isolated from furunculosis outbreaks and were thus assumed to be virulent (Manuscript III).

Chapter 2: Furunculosis

2.1 Historical background

Furunculosis is now spread worldwide, though the first time furunculosis was observed and documented among fish was in 1894 by Emmerich and Weibel. They observed swellings resembling boils as well as ulcerative lesions in brown trout (*Salmo trutta*) at a German freshwater hatchery. Another observation of Emmerich and Weibel was that the brown trout were held in low-quality water before being transported to a farm where other fish also became infected. Through experiments Emmerich and Weibel also discovered that by either intramuscularly injecting fish or adding culture to the water tank, the fish could become infected. Cohabitation experiments gave the same results, however, when examining healthy fish from these experiments, the bacterium was not found.

After the initial description by Emmerich and Weibel (1894), furunculosis was believed to be a hatchery associated infection until the studies of Plehn (1911) showed that furunculosis was also present among wild trout in Germany and others also observed the infection in several countries all over the world, including Great Britain who suffered great losses (Fuhrman, 1909; Pittet, 1910; Surbeck, 1911; Arkwright, 1912; Mettam, 1915; Christensen, 1980). In the United States, furunculosis was first described by Marsh (1902) at hatcheries in Michigan. Shortly thereafter, the infection was found in numerous salmon and trout hatcheries throughout the United States (Fish, 1937; Smith, 1942). The origin of furunculosis in the United States is uncertain, though the general theory is that either it was brought along with brown trout from Germany or it spread from rainbow trout farmed in the Western part of the United States (Fish, 1937). Signs of furunculosis were also seen in several fish species in Canada by Duff and Stewart (1933) and various trout farms in Japan (Furunculosis comittee, 1933).

In Denmark furunculosis was first described in the 1950s at freshwater rainbow trout farms by Rasmussen (1964). In parallel with this discovery, a massive expansion in rainbow trout production started that continued its growth even further as production became established in seawater in the 1970s (Christensen, 1980). Moreover, a Bayesian temporal tree based on SNP analysis of 101 sequenced *A. salmonicida* showed that there have been four main introductions of *A. salmonicida* in Denmark, two of which occurred approximately the same time as the first expansion in rainbow trout production (~ 1950) and the other two during the second expansion in seawater (~ 1970) (Manuscript III). At present, it is in the seawater production during elevated temperatures that furunculosis is of great concern and causes huge financial losses (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000; Pedersen et al., 2008).

2.2 Clinical signs of disease

Fish infected with *A. salmonicida* do not necessarily show any clinical signs of disease; however, when fish become stressed or are compromised in some way, such that their immune system is lowered and a favorable condition within the fish is created for the pathogen, the infection can spread throughout the body and clinical signs can become visible (Cipriano et al. 1997; Hiney et al., 1997;

Hiney and Olivier, 1999; Austin and Austin, 2007; Noga, 2010). Typical clinical signs of the infection can include lethargy, lack of appetite, skin hyperpigmentation, boils and/or ulcers on the skin, lesions, internal hemorrhaging, enlargement of the spleen, septicemia and anemia (Fig. 2) (McCarthy, 1977; Ferguson and McCarthy, 1978; McCarthy and Roberts, 1980; Hiney et al., 1997; Hiney and Olivier, 1999; Austin and Austin, 2007; Noga, 2010).

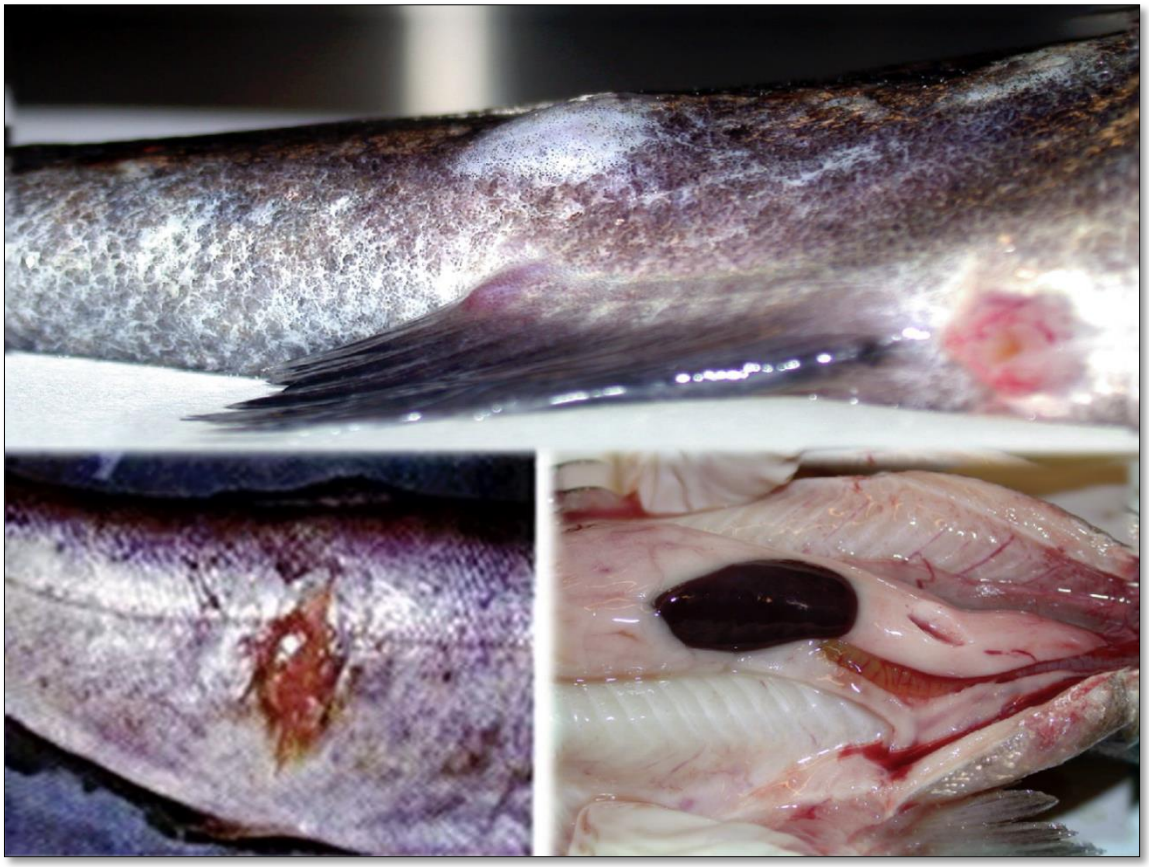

Figure 2. Rainbow trout with signs of furunculosis. At the top: Boil and ulcer on the skin (photo by Morten Sichlau Bruun). To the left: Ulcer on the skin (Christensen, 1980). To the right: Enlargement of the spleen and hemorrhaging from internal organs (photo by Morten Sichlau Bruun).

2.3 Antibiotic treatment

In Denmark the antibiotics used in aquaculture have been sulfadiazine, trimethoprim, oxytetracycline and furazolidone (Dalsgaard et al., 1994) and since 1986, the only antibiotics licensed for use in aquaculture have been sulfadiazine/trimethoprim and oxolinic acid. The prevalence of antibiotic resistance genes (ARGs) among *A. salmonicida* in Denmark has been low, 5% in the study by Dalsgaard et al. (1994) and 9% in Manuscript III. Nevertheless, repeated treatment with antibiotics has proven to have many drawbacks, including induction of drug resistance in microorganisms, suppression of the immune system in fish, accumulation of residues in the fish, sediment and surrounding environment of the fish farms (Rijkers et al., 1981; Jacobsen and Berglind, 1988; Björklund et al., 1990; Aoki, 1997; Sørum, 1998; Sørum, 1999; Muziasari *et al.*, 2014). One major threat posed regarding antibiotic treatment is the ability of various genetic elements such as ARG

carrying plasmids (R plasmids) and integrons to disseminate multiple transferable ARGs (Aoki, 1997; L'Abée-Lund and Sørum, 2001; Berglund, 2015).

In the study by L'Abée-Lund and Sørum (2001), *A. salmonicida* and other bacteria originating from different locations around the world were investigated for the presence of a class 1 integron. Along with the integron, several ARGs were found: *aadA2*, *dfr16*, *aadA1*, *dfrIIc*, *qacG*, *orfD*, *tetA* and *tetE*, indicating that not only do class 1 integrons facilitate antibiotic resistance in marine environments, but also that ARGs can be transmitted between bacteria in various environments, since the found ARGs cassettes have also been associated with humans (L'Abée-Lund and Sørum, 2001). In agreement, Muziasari et al. (2014) found class 1 integrons and ARGs *sul1*, *sul2* and *dfrA1* in the sediment from farms located in the northern Baltic Sea and these same three ARGs, along with *aadA2* and *aadA1*, were also found in Danish *A. salmonicida* isolated from furunculosis outbreaks (Manuscript III). Kadlec et al. (2011) moreover found both class 1 integrons and ARGs against sulfonamide, trimethoprim and other antibiotics among *Aeromonas* species from Germany, where the only antibiotic therapy of fish is a combination of the two mentioned antibiotics.

The greatest concern with broad host range conjugative plasmids is that they can transfer ARGs across different bacterial genera and similar R plasmids have been isolated from separate ecological niches and across different environments (Sørum, 1998; L'Abée-Lund and Sørum, 2000; Sørum et al., 2003; Smillie et al., 2010). Sørum (1998) reported that after only 24 hours of mating between a fish pathogenic atypical *Aeromonas* carrying an R plasmid and *Escherichia coli*, the plasmid was directly transferred to every second *E. coli* cell. Direct transfer of the R plasmid from the atypical *Aeromonas* to human pathogens like *Salmonella enteritidis* and *Salmonella typhimurium* was also possible (Sørum, 1998). The atypical *Aeromonas* was also believed to be the origin of an R plasmid in *A. salmonicida* from a furunculosis outbreak (Sørum, 1998). Direct transfer of ARGs from pathogenic *A. salmonicida* to *E. coli* cells was also reported in the study by (Aoki et al., 1971). In the whole genome sequencing (WGS) study, none of the 101 *A. salmonicida* that were sequenced harbored any of the five investigated R plasmids (Manuscript III). However, eight *A. salmonicida* that also harbored multiple ARGs did show coverage (< 60%) of at least one of the R plasmids, indicating they could have acquired ARGs from the plasmids in the past through horizontal gene transfer and then subsequently lost the plasmid.

2.4 Vaccination

Unlike treatment with antibiotics, one does not have to worry about the bacterial pathogens developing resistance against vaccinations (Vinitnantharat et al., 1999), who provide a better alternative for future control of furunculosis. Immunization of fish against furunculosis by vaccine administration was already introduced experimentally in 1937, however, not until the early 1990's successful implementation of oil-adjuvanted vaccines in salmon aquaculture has there been made great advances in this field of research (Midtlyng, 1997). Fish can be immunized orally or by immersion or injection, though oral and immersion vaccines are less stressful for the fish than injection and would be preferred if their protection level would equal the one produced by injection (Vinitnantharat et al., 1999). Unfortunately, although many attempts have been made to produce an oral vaccine against furunculosis, thus far all have exhibited inconsistent antibody response and

protection (e.g. Krantz et al., 1964; Spence et al., 1965; Midtlyng et al., 1996), which in part could be caused by the fact that the vaccine components are poorly retained when administered orally (Press et al., 1996). Despite initially showing promising results (Cipriano et al., 1983; Johnson and Amend, 1984; Rodgers, 1990), immersion vaccines have also proved to be inadequate for use in aquaculture due to lack of long-term protection (Midtlyng et al., 1996; Midtlyng, 1997).

Over time, handling techniques improved for injection vaccines and automatic equipment, manuals and instruction videos for training became available, making it possible for injection vaccines to be administered on a large scale (Eithum, 1993; Midtlyng, 1997). Intraperitoneal injection of vaccines with oil adjuvant such as mineral oil, moreover induced much greater and longer protection compared to oral and immersion vaccines, making this vaccine administration superior to the others (Midtlyng, 1996; Midtlyng et al., 1996; Midtlyng, 1997). Even though numerous side effects for oil adjuvants have been observed, including lesions, pigmentation, granulomatous inflammation in the liver, autoimmune reactions and intra-abdominal adherences, it is still recommended to use this administrative method to minimalize loss of fish due to disease (Midtlyng, 1996; Midtlyng et al., 1996; Midtlyng, 1997; Håstein et al., 2005; Koppang et al., 2008; Satoh et al., 2011). The results of Mutoloki et al. (2006) using Atlantic salmon moreover showed that the combination of antigen and oil adjuvant is crucial and that it is their combined effect that is responsible for induction of a strong inflammatory reaction in the fish, thus highlighting the importance of choosing the correct antigen in order to develop an effective vaccine. Several antigen candidates have been suggested for stimulating early inflammatory reactions against *A. salmonicida*, which among others includes the A-layer, LPSs, IROMPs and ECPs (Midtlyng, 1997; Mutoloki et al., 2006).

The subspecies *A. salmonicida* is known to be very homogenous and this includes its virulence related A-layer proteins and LPSs located on the surface of the bacterium (Bjørnsdottir et al., 1992; Arnesen et al., 2010), which was also observed in Manuscript III. The combination of high similarity and location makes them very good antigen candidates and a positive correlation between vaccinating with A-layer proteins and protection by the immune system has been observed (Lund et al., 2003a; Arnesen et al., 2010). Research involving *A. salmonicida* IROMPs has shown that they also could have a potential as antigens included in vaccines, due to their *in vitro* bactericidal effect on both A-layer negative and positive *A. salmonicida* strains and *in vivo* protection of Atlantic salmon (Bricknell et al., 1999; O'Dowd et al., 1999). ECPs of *A. salmonicida* are already part of oil adjuvant vaccines against furunculosis, however, their contribution to the vaccine protection remains uncertain as studies using ECPs or their extracts as antigen showed varying results (Cipriano, 1982; Cipriano and Pyle, 1985; Prost, 2001). In fact, ECPs might not be important for inducing a protective immune responses (Lund et al., 2003a). There are even studies indicating that inclusion of ECPs in vaccines could have an adverse effect (Hirst and Ellis, 1994; Midtlyng et al., 1996; Lund et al., 2003a).

2.5 Transmission

Furunculosis and its causative agent *A. salmonicida* have been investigated ever since the first discovery of furunculosis in 1894 by Emmerich and Weibel. Nevertheless the topic of transmission of *A. salmonicida* remains to be resolved. The primary focus and problem regarding transmission has

been carrier fish, whose existence was already indicated by several studies shortly after the discovery of furunculosis itself, by illustrating that the presence of *A. salmonicida* in fish does not necessarily lead to the development of furunculosis (e.g. Plehn, 1911; Mettam, 1915; Horne, 1928). Fish are thought to be capable of being infected with *A. salmonicida* for up to several months without showing any clinical signs of disease, in which time the infection is often said to be in a *latent* or covert phase and the fish are said to be "carriers" of the bacterium (Hiney et al., 1997).

Carrier fish have been recognized to play a significant role in the transmission of *A. salmonicida*, due to the ability of fish being able to shed bacteria in their surroundings and *A. salmonicida* being able to survive in water without a host (McCarthy, 1980; Rose et al., 1989b; Rose et al., 1990; Hastein and Lindstad, 1991; Nomura et al., 1992; Smith, 1992; Morgan et al., 1993; Nomura et al., 1993; Ogut and Reno, 2005). In a cohabitation study where Chinook salmon (*Oncorhynchus tshawytscha*) were injected with *A. salmonicida* and placed together with uninfected fish for ten days, prevalence of the bacterium among the initially healthy recipient fish was as high as 75% (Ogut and Reno, 2005). Mortality related to disease of the recipient fish moreover surpassed 50% and both bacterial concentrations in the water number of infected fish increased with time (Ogut and Reno, 2005). Rose et al. (1989b) studied Atlantic salmon experimentally infected with *A. salmonicida* in seawater and found bacteria were shed from dead and moribund fish at a high rate of 10^5 - 10^8 colony-forming units (CFU) per fish per hour.

Even though *A. salmonicida* initially has been thought of as an obligate pathogen not being able to survive in water without presence of fish (Popoff, 1984), others such as McCarthy (1980) found that the bacterium could survive up to 8 days. This was supported by the study of Nomura et al. (1992), where *A. salmonicida* was monitored in various type of water. In sterilized fresh water the bacterium could survive for sixty days while in non-sterile water, only around four days (Nomura et al., 1992). Survival in sea water was moreover shorter than in fresh water, though Nomura et al. (1992) concluded that *A. salmonicida* survival time in water was enough to infect other fish via this route. In a 21-day study of *A. salmonicida* in untreated lake water, Morgan et al. (1993) detected the bacterium in water samples by cultivation on TSA plates and with polymerase chain reaction (PCR) DNA amplification. Another interesting finding regarding survival of *A. salmonicida* in water is related to the increased hydrophobicity caused by the possession of the A-layer (Enger, 1997), whereby the A-layer positive *A. salmonicida* would become concentrated at the water surface and thus especially be a concern for the farms that use spray aeration. Nevertheless, even though no water samples were investigated, no gills were found positive from 20 fish showing no signs of disease that were sampled from three freshwater farms and one seawater farm in Denmark (Manuscript II).

Usually carrier fish start showing clinical manifestations of furunculosis when they become stressed, such as during rise in water temperature (Plehn, 1911; Bullock and Stuckey, 1975; McCarthy, 1977; Bernoth et al., 1997; Noga, 2010). This scenario is believed to be the key for transmission of *A. salmonicida* and cause of the furunculosis outbreaks among Danish fish farms. Initially, furunculosis was first observed by Rasmussen (1964) in freshwater, however, today outbreaks occur in seawater during periods of high temperature and especially causes great losses in sea reared rainbow trout production (Dalsgaard and Madsen, 2000; Pedersen et al., 2008). The theory is that fish at Danish

freshwater farms could be carriers of *A. salmonicida* and transfer the bacterium with them to the seawater farms, where the fish become stressed and develop the disease (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000; Pedersen et al., 2008). This theory is in agreement with results from Manuscript II, where most of the investigated fish were from a batch that was followed from freshwater to seawater. Some of these fish that showed no signs of disease at the freshwater farms were positive for *A. salmonicida* with quantitative real-time PCR (real-time PCR) and when transferred to a seawater farm, two furunculosis outbreaks occurred at the farm later on during high temperatures.

A different suggestion of *A. salmonicida* transmission has been that the bacterium is transmitted vertically (Wichardt et al., 1989). However, this was disputed through an extended study carried out by Bullock and Stuckey (1975), who followed carrier and artificially infected brood fish without finding any signs of vertical transmission. This notion was supported by McCarthy (1977), instead it was proposed that transmission is related to both contaminated equipment and infected fish. McCarthy (1977) and Rose et al. (1989b) also investigated transmission by feed through the gastrointestinal track, though without yielding any convincing results. Further proposed routes of transmission includes e.g. ciliated protozoans, who seemingly enhanced survival of *A. salmonicida* when the bacterium was co-cultured with it (King and Shotts, 1988).

2.6 Route of entry and colonization site(s)

Although several suggestions have been made, there is currently still a great deal of uncertainty regarding both the route of entry and primary colonization site(s) of *A. salmonicida* and especially in carrier fish. The fins, gills, mucus layer as well as openings such as wounds have been proposed as possible entry sites for *A. salmonicida* (Hiney et al., 1997). In agreement, in an experimental study, artificially wounded Atlantic salmon infected with *A. salmonicida* showed higher mortality than salmon without the wounds (Svendsen and Bøgwald, 1997). Svendsen and Bøgwald (1997) further concluded that *A. salmonicida* can also adhere and most likely penetrate the mucous and skin of Atlantic salmon. The fins were suggested as a possible entry site for *A. salmonicida* by Hiney et al. (1994), who detected *A. salmonicida* in fins of pre-smolt Atlantic salmon. In another study with Atlantic salmon, the gills were indicated as a possible portal of entry into the fish (Svendsen et al., 1999). These findings are in congruence with Tatner et al. (1984) who investigated *A. salmonicida* infection in rainbow trout as well with results of Manuscript I, where the suggested main attachment sites for *A. salmonicida* after an experimental immersion were the dorsal and pectoral fins and gills.

In regards to possible colonization sites, there are several possible organs that have been investigated and proposed as the most probable site for *A. salmonicida* colonization. The first organ to be called the primary colonization site was the kidney (McCarthy, 1977; Popoff, 1984; Hiney et al., 1997). Though, *A. salmonicida* was also found in the gut as early as 1911 by Plehn. The gut was even the sole organ where *A. salmonicida* was found in naturally infected fish without showing clinical signs of disease by Willumsen (1990). By use of enzyme-linked immunosorbent assay (ELISA) *A. salmonicida* was also found in the gut by Rose et al. (1989b) and by use of PCR in the study of Gustafson et al. (1992). The bacterium was also found in the gut of experimentally infected rainbow trout in Manuscript I. Other organs such as the liver, heart, spleen or blood have also been used in

many studies as sampling sites for *A. salmonicida* (e.g. Daly and Stevenson, 1985; Cipriano, 1997; Hiney et al., 1997; Svendsen et al. 1999; Beaz-Hidalgo and Figueras, 2012). The spleen, along with the kidney, were also both positive for *A. salmonicida* by bacterial culturing in all experimentally infected fish in Manuscript I.

2.7 Susceptibility of fish species

Initially furunculosis was believed to be an exclusive disease of salmonids. Since then it has become known that *A. salmonicida* can also infect other fish species and other aquatic animals in freshwater and seawater e.g. catfish, carp, turbot, American eel, goby and wrasse (Bernoth et al., 1997). It has also become apparent that susceptibility to furunculosis varies among the host species (e.g. Plehn, 1911; Fish, 1937; McCarthy, 1977; Ellis and Stapleton, 1988; Perez et al., 1996). The first to document that species have different susceptibilities to *A. salmonicida* was Plehn (1911), who conducted experiments with infected rainbow trout and brown trout. The key result was that infected rainbow trout could remain unaffected and when placed in the same tank with brown trout and temperature was raised, brown trout developed disease and died, while the rainbow trout still remained unaffected (Plehn, 1911).

In general, fish belonging to the family Salmonidae are thought to be the most susceptible to furunculosis (McCarthy, 1977). Especially brown trout, brook trout (*Salvelinus fontinalis*) and Atlantic salmon have shown to be highly susceptible, while rainbow trout seemed to be more resistant as they needed to be wounded in a bath experiment before showing any signs of disease (McCarthy, 1977). The high degree of resistance which rainbow trout seem to possess against furunculosis compared to other farmed fish species was also illustrated by Cipriano et al. (1994a). Though it has to be mentioned that in their study, McCarthy and Roberts (1980) have argued that the presumed high susceptibility of salmonids to furunculosis might simply be related to the high degree of research that has been done on this family of fish due to their value as farmed fish.

Difference in susceptibility to furunculosis has been related to their immune system activity and especially their varying mucosal activity that is one of the main physical barriers and contains bioactive molecules such as lysosomes and other bacteriolytic enzymes (e.g. Cipriano and Heartwell, 1986; Cipriano et al., 1992; Cipriano et al., 1994a; Svendsen and Bøgwald, 1997). Teleost (bony) fish in general do exhibit a variation in their immune system wherein mucosal activity against pathogens is included (Dickerson, 2009). In agreement, a study by Cipriano and Heartwell (1986) showed that the fish species' mucus antibacterial activity directly correlated with their resistance towards furunculosis. This is further supported by results from Manuscript I, where the skin was not among the suggested primary attachment sites of *A. salmonicida* of rainbow trout that are known for their high resistance against *A. salmonicida*. Svendsen and Bøgwald (1997) also showed that mortality was higher for Atlantic salmon with an impaired skin mucous layer versus salmon with an intact mucous whereby it is indicated that skin mucous likely plays a role in the defense against *A. salmonicida*. Moreover, Cipriano et al. (1992) argued that it appears that several fish species actually lack an effective mucous protection layer against furunculosis.

2.8 *In vivo* imaging

One approach that could help elucidating the uncertainties about transmission, route of entry and colonization of *A. salmonicida*, as well as shed more light on the variation of species susceptibility to *A. salmonicida*, is tracking *A. salmonicida in vivo* in living fish. Tracking of *A. salmonicida* has been previously done by labeling the bacterium with radioactive isotopes (Svendsen et al., 1999). This resulted in findings such as a higher mortality in fish with artificial wounds and reduced epidermal mucus and also highlighted the importance of sampling time and the location of the bacterium. As an example, *A. salmonicida* was found in the blood after two hours, but not after 24 hours (Svendsen et al., 1999) .

In recent years much progress has been made regarding *in vivo* imaging and the two types that are most commonly used are bioluminescent and fluorescent reporters (Troy et al., 2004). Fluorescent proteins include the green fluorescent protein (GFP) (Chishima et al., 1997; Bouvet et al., 2002; Winnard et al., 2006) and DsRed (Baird et al., 2000; Dietrich and Maiss, 2002; Troy et al., 2004), however, there are numerous colors and near-infra red fluorescent dyes to choose from (Weissleder et al., 1999; Olenych et al., 2007; Day and Davidson, 2009; Filonov et al., 2011). Genes used in Bioluminescence imaging (BLI) originate from various luciferase proteins in bacteria, firefly, click beetles, and Renilla and the following components are involved in the light emission reaction: luciferase, luciferin, oxygen, and ATP (Troy et al., 2004). Although firefly and red click beetle luciferases are preferred in some studies due to their longer wavelength emission, bacterial luciferase is the only luciferase that does not require an injection of luciferin, which is a compound consisting of a long-chain aldehyde and a reduced riboflavin phosphate ($FMNH_2$), in order to initiate the light-producing reaction (Troy et al., 2004). This is because the bacterial *lux* operon (*lux*CDABE) consists of five genes that encode both the luciferase enzyme and the aldehyde substrate (Fig. 3), while $FMNH_2$ is provided from the electron transport chain present in all bacteria (Troy et al., 2004; Lin and Meighen, 2009).

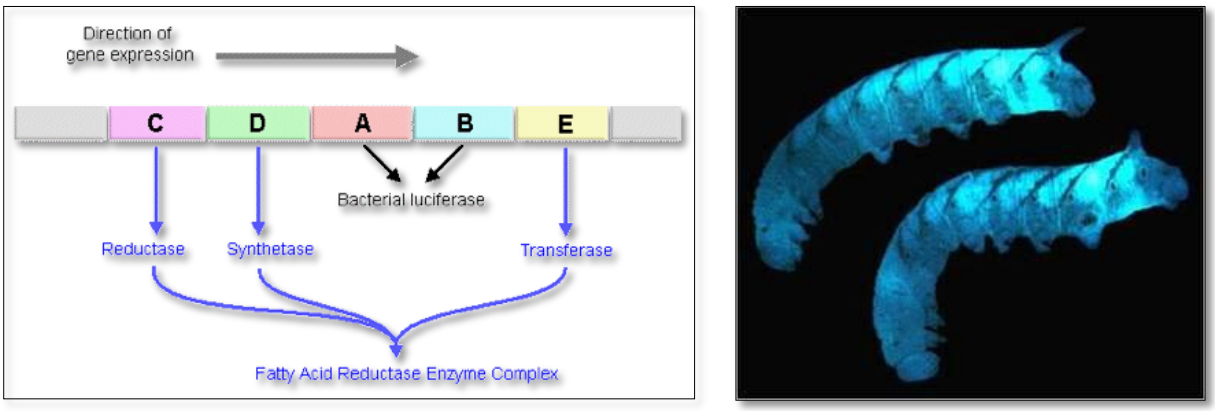

Figure 3. To the left: An illustration of genes of the bacterial *lux* operon. Genes *luxC*, *luxD*, and *luxE* code for a fatty acid reductase, a multicomplex enzyme that continuously supplies and regenerates the aldehyde substrate, while the genes *lux*A and *lux*B code for luciferase (Lin and Meighen, 2009). To the right: Bioluminescent *Photorhabdus luminescens* bacteria inside a nematode worm (Byrne, 2011).

When comparing the advantages and disadvantages of BLI versus fluorescence imaging, one has to take into consideration the type of imaging experiment one wants to execute. Luciferase proteins are also short lived, while fluorescence proteins are able to reside in the cell for hours (Burns et al. 2001; Troy et al. 2004), making fluorescence proteins more advantageous reporters for long term studies. Though, fluorescence proteins can take hours to become functional, while luciferase proteins mature rapidly following expression, making luciferase proteins more suited for shorter studies (Burns et al. 2001; Troy et al. 2004). Fluorescence imaging also has high "background noise", meaning the animal itself emits fluorescence that interferes with the actual signal one is trying to visualize, while this kind of background noise is scarce in BLI (Burns et al. 2001; Troy et al. 2004). The background noise emitted by rainbow trout tissue in the immersion experiment conducted in Manuscript I was also scarce and could not be visualized. Luciferase proteins also have higher sensitivity and lower toxicity than fluorescence proteins (Burns et al. 2001; Troy et al. 2004). Bacterial luciferase is also an excellent choice for non-invasive studies, due to its ability of emitting light continuously without the need of adding any other substrate by e.g. an injection (Troy et al., 2004). However, although BLI three-dimensional techniques are available commercially (Virostko et al., 2008), BLI is usually used as a two-dimensional imaging technique and the spatial resolution is low compared to fluorescence imaging, making it difficult to separate photons produced by infected cells in two adjacent sites (Hutchens and Luker, 2007).

BLI has also been applied for monitoring *A. salmonicida* in Atlantic salmon by Ferguson et al. (1998). However, in these experiments an exogenous addition of the aldehyde substrate was needed in order to catalyze the light reaction and thereby visualize the bacterium. The incorporation of the luciferase genes into *A. salmonicida* moreover significantly lowered the virulence of the bacterium (Ferguson et al., 1998). Nevertheless, the experiment showed that *A. salmonicida* was shed from moribund and dead fish in the water column and were able to infect cohabitant fish, where the bacteria were mostly found in the gills and skin mucous. In recent studies of the fish pathogen *Edwardsiella ictaluri* by Karsi et al. (2006) and Menanteau-Ledouble et al. (2011), a bacterial luciferase *lux* operon from *Photorhabdus luminescens* (Fig. 3) that emits light continuously by itself was used, enabling them to follow fish over several time points without the need for euthanization until the end of the experiments. Méndez and Guijarro (2013) also used this *lux* operon to successfully trace dissemination of *Yersinia ruckeri* in rainbow trout.

2.9 Manuscript I

Due to the fact that our focus for this study lay on the initial stages of the infection by *A. salmonicida*, meaning the route of entry and initial dissemination of *A. salmonicida* in fish, we choose to employ the same bacterial luciferase *lux* operon as the above mentioned authors in order to track *A. salmonicida* by *in vivo* BLI. This was done by transforming a highly virulent Danish *A. salmonicida* with a plasmid vector containing both GFP genes and the bacterial luciferase *lux* operon, which was subsequently used in immersion experiments where fish were followed over a 24-hour period. Although only luciferase was visualized in the immersion experiments, GFP enabled a practical way of visualizing the bacterial colonies through use of a fluorescence microscope. Results of these experiments showed that probable colonization sites of *A. salmonicida* are the gills and the dorsal and pectoral fins. The bacteria then progressed through the internal organs and seem to exit

via the anal opening. Modifications of this method are needed in order to attain more comprehensive knowledge regarding the route of entry and dissemination of *A. salmonicida*, nevertheless, this method does provide a possible tool for visualizing colonization of *A. salmonicida* and other bacterial pathogens in fish, as well as study host-pathogen interactions.

Chapter 3: Detection

3.1 Detection of carrier fish

The main problem regarding identification of carrier fish has been the difficulty of detecting *A. salmonicida* within them (Bullock and Stuckey, 1975; Dalsgaard & Madsen 2000). Isolating *A. salmonicida* from fish showing signs of diseases has usually been done by bacterial culturing and the bacterium can also be detected in the fish by methods like histopathology, ELISA and PCR (Rose et al., 1989a; Gustafson et al., 1992; Dalsgaard and Madsen, 2000; Austin and Austin, 2007; Beaz-Hidalgo et al., 2013). There are several explanations for the struggle with detection of *A. salmonicida* and especially with bacterial culturing, which has been the most frequently used method for *A. salmonicida* detection throughout the years (Hiney et al., 1997; Austin and Austin, 2007). One of the two main notions is that the amount of *A. salmonicida* within the carriers might be too low for colonies being able to grow (Hiney et al., 1994). Another possibility is that the bacterium within carrier fish can be present in a non-culturable state, i.e. they are viable but non-culturable cells (VBNC) (Morgan et al., 1993; Ferguson et al., 1995; Năşcuţiu, 2010). The presence of VBNC *A. salmonicida* has, however, been a controversial subject due to the scepticism towards being able to revive these cells after their non-culturable state. Presence of VBNC *A. salmonicida* in fish would, nevertheless, correlate with the scenario in Denmark, where it has not been possible to detect the bacterium by culturing in rainbow trout from freshwater farms, but furunculosis outbreaks still occur during elevated temperatures after these fish are transferred out to seawater farms (Dalsgaard & Madsen 2000; Pedersen *et al.* 2008).

In order to improve detection of *A. salmonicida* in carrier fish, various methods have been applied including heat stress of fish, injection of corticosteroid into fish and pre-enrichment steps for bacterial culturing. The first to employ a stress test in order to discover carriers was Plehn (1911). In an experimental infection with *A. salmonicida*, fish infected with the bacterium were stressed by temperature increase, whereby some fish started showing signs of disease and soon thereafter died (Plehn, 1911). Though, Bullock and Stuckey (1975) reported that although heat stress by increase of temperature to 18^{o}C did cause clinical signs of the disease and subsequently high mortality among carrier fish, detection from the fish still remained very low and the best way to increase detection in carriers was a combination of corticosteroid injection and heat stress, termed stress-inducible furunculosis (SIF) tests (Smith, 1991; Cipriano et al., 1997). The drawback of this method is that injected fish have to be held in heated tanks for about two weeks. Even though the method was modified in 1977 by McCarthy, the SIF tests are still time consuming and also require sacrifice of many fish for obtaining statistical significance (Hiney et al., 1994). Nevertheless, the high reliability of this method has consequently made it the primary examination method of salmonids in European aquaculture (Smith, 1991). In the study by Cipriano et al. (1997), performance of a SIF test was compared to another method suggested for usage of enabling detection of carriers, namely employing pre-enrichment steps before culturing (Daly and Stevenson, 1985; Cipriano et al., 1997). Though, statistical analysis revealed that the SIF test was more reliable than both pre-enrichment and direct culturing (Cipriano et al., 1997). One factor, however, needs to be considered when applying SIF tests to salmon, which is timing the test close to smoltification (Scallan and Smith, 1993).

3.2 PCR

In order to avoid the SIF tests, yet maintain the possibility of detecting carriers, molecular methods such as PCR assays have been developed for detecting *Aeromonas salmonicida* subspecies (Gustafson et al., 1992; Hiney et al., 1992; O´Brien et al., 1994; Mooney et al., 1995; Byers et al., 2002a, b; Altinok et al., 2008; Beaz-Hidalgo et al., 2008b). PCR has proven to be a powerful tool for amplification of nucleic acids, whereby a DNA sequence selected for amplification is exponentially increased in repeated cycles of synthesis by a thermostable DNA polymerase and two oligonucleotide primers that each hybridize to one strand of the double-stranded target DNA (Saiki et al., 1985; Saiki et al., 1988). This is usually followed by visualization of the amplified DNA by gel electrophoresis and a known DNA probe is used for determining size of the amplicon.

One of the first *Aeromonas salmonicida* subspecies specific PCRs was developed by Hiney et al. (1992), based on a DNA fragment (GenBank accession number X64214) of a 6.4 kb *A. salmonicida* cryptic plasmid. Sensitivity of detection for pure culture was two cells (Hiney et al., 1992) and no false positives were amplified when tested with numerous typed and clinical isolates of related aeromonads and other bacterial genera (Hiney et al., 1992). This target DNA was later used by multiple authors (e.g. Morgan et al., 1993; O´Brien et al., 1994; Byers et al., 2002a, b; Altinok et al., 2008) for detecting *Aeromonas salmonicida* subspecies from various fish tissues, feces and water. In all the studies no false positives were obtained by testing non-target bacterial DNA, however, not all *Aeromonas salmonicida* subspecies were identified. This is because the cryptic target plasmid that now has been identified as *A. salmonicida* plasmid pAsal1 sequenced by Fehr et al. (2006) (GenBank accession number AJ508382), is not universally present in all *A. salmonicida* (Nielsen et al., 1993; Attéré et al., 2015) and can be lost by culturing at above 22 - 25^{o}C (Daher et al., 2011; Tanaka et al., 2012; Attéré et al., 2015). Though, in the 99 sequenced *A. salmonicida* that were isolated from furunculosis outbreaks in Denmark in Manuscript III, only 52% seemed to harbor pAsal1 and all were grown at 20^{o}C.

Another target gene that has been used by more than one author is the *vapA* gene, which encodes the A-protein of the A-layer and was initially used as a PCR target by Gustafson et al. (1992). Sensitivity for detecting *Aeromonas salmonicida* subspecies in fish tissue was 10 CFU mg^{-1} and only 1 CFU ml^{-1} in pure culture. Nevertheless, it was concluded that enrichment steps were necessary for detecting potential carrier fish in order to avoid false negatives. As with the pAsal1 target, not all bacterial isolates were identified with use of the *vapA* target due to mutations in the *vapA* gene (Gustafson et al., 1992; Byers et al., 2002b). The pAsal1 target and *vapA* target were combined in the study by Byers et al. (2002b) and they correctly identified 93% and 94% of the *Aeromonas salmonicida* subspecies respectively. When used together, the two PCR assays identified 99% of the isolates (Byers et al., 2002b), though in another study conducted by the same authors, it was concluded that bacterial culturing was more reliable for detecting carrier fish than the PCR method and if PCR were to be used, pre-enrichment steps would be necessary (Byers et al., 2002a).

The chromosome encoded *fstA* gene has also been used as a PCR target in the study of Beaz-Hidalgo et al. (2008b). What separated this PCR from the others, according to Beaz-Hidalgo et al. (2008b), was that blood and mucus were used as sampling sites, making the PCR assay a non-destructive

diagnostic tool. Detection limit for experimentally infected mucus and blood samples were 2.5 x 10^2 and 1.5 x 10^5 CFU ml^{-1} respectively. It is thought that the low sensitivity in blood samples was caused by heparin or other blood component interference that are able to compete with bacterial DNA in the assay. Nevertheless, when tested on potential carriers, 31 wild salmon with no signs of disease, six salmon were detected by the PCR while the culture method only detected one fish (Beaz-Hidalgo et al., 2008b).

3.3 Real-time PCR

The conventional method of PCR revolutionized molecular techniques in science, nevertheless, the fact remains that this method is laborious, difficult to automate, needs enrichment steps for enabling detection of carriers and is usually semi-quantitative at best (Kubista et al., 2006; Bustin et al., 2012). This is because conventional PCR is restrained by reagents and reaches a plateau where the product amount cannot be increased any longer (Bustin et al., 2012). This plateau varies from assay to assay and since the PCR is an endpoint assay analyzed after the reaction has reached its linear phase, the gel electrophoresis analysis shows roughly the same amount of DNA that was produced by the end of the PCR reaction regardless of what the initial amount of DNA was (Bustin et al., 2012). Although the same amplification principles apply to the real-time PCR method, as the name reveals, this method monitors the amplification process in "real time" and can be used for precise quantitative analysis by fluorescent reporter molecules (Higuchi et al., 1992; Kubista et al., 2006; Bustin et al., 2012). For real-time PCR, there are also different detection chemistries to choose from and the two major ones are either an intercalating dye such as SYBR green (Morrison et al., 1998) or a hydrolysis probe such as Taqman (Gibson et al., 1996). Both chemistries have their advantages and disadvantages though they share the same design, which is to generate fluorescence during the PCR reaction that is monitored in "real time". Apart from enabling quantitative application, real-time PCR is also convenient, robust, simple, fast, sensitive and adapted to high throughput analysis; as a consequence it has become the most widely used molecular technique (Kubista et al., 2006; Bustin et al., 2012)

Real-time PCR has been used in several studies to detect *Aeromonas salmonicida* subspecies directly from fish tissue (e.g. Balcazar et al., 2007; Goodwin and Merry, 2009; Keeling et al., 2012; Gulla et al., 2015). In the study by Balcazar et al. (2007) a real-time PCR assay design was developed, which combined low costs with high sensitivity (Balcazar et al., 2007; Nazarenko et al., 2002a, 2002b). The assay was based on Light Upon eXtension (LUX) primer probes originally described by Nazarenko et al. (2002a, 2002b). The primers targeted the same *A. salmonicida* DNA sequence (gene *aopP*) located on the pAsal1 plasmid as originally developed by Hiney et al. (1992) for conventional PCR. In agreement with all previous PCR studies that had implemented this target, Balcazar et al. (2007) did not find any false positives. Though, on the contrary to the bulk of the previous studies, Balcazar et al. (2007) did obtain 100% correct identification of 16 isolates of both typical and atypical *Aeromonas salmonicida*. Balcazar et al. (2007) stressed that the reason behind this result is that the isolates used in his study were all from various disease outbreaks indicating that the presence of pAsal1 in the bacterium could be related to virulence of the bacterium. This hypothesis is supported by research of Goodwin and Merry (2009) who also used the same target for a real-time PCR assay to detect atypical *Aeromonas salmonicida* and also obtained 100% amplification of this species. In

our Manuscript II, all of the 20 tested *A. salmonicida* isolates from furunculosis outbreaks were also correctly identified by the real-time PCR originally developed by Balcazar et al. (2007). Nonetheless, the theory was later disputed by our research where 99 Danish *A. salmonicida* isolated from furunculosis outbreaks, were subjected to WGS and it was found that the AopP protein sequence encoded on pAsal1 was missing in 50% of the *A. salmonicida* isolates (Manuscript III).

In the two real-time PCR assays developed by Keeling et al. (2012) and Gulla et al. (2015) the same target sequence (gene *vapA*) was used as originally applied for conventional PCR by Gustafson et al. (1992). The assay by Keeling et al. (2012) was based on a molecular beacon and the assay was found to have 100% analytical specificity and an analytical sensitivity of 5 ± 0 fg in pure culture and 2.2 x 10^4 ± 1 x 10^4 CFU g^{-1} for kidney tissue. Though, with enrichment steps as used in earlier research, the sensitivity for tissue increased to 40 ± 10 CFU g^{-1}. This assay was later modified by Gulla et al. (2015) for detecting atypical *Aeromonas salmonicida* from head kidney of cleaner fish in Norway, due to existence of multiple types of the A-layer caused by a single base variation resulting in a mismatch with the forward primer developed by Keeling et al. (2012). This variation in the A-layer was most likely responsible for why 6% of the *Aeromonas salmonicida* subspecies investigated in the study by Byers et al., (2002a) were not amplified by the *vapA* primers. After this modification, Gulla et al. (2015) obtained 100% analytical specificity of all presently recognized A-layer types of this species and a sensitivity of 7 - 8 bacterial genomes in pure culture and 1.1 x 10^3 CFU mL^{-1} in tissue.

3.4 Manuscript II

In order to develop a molecular method that would enable specific and highly sensitive detection directly from multiple fish tissue (even potential carrier fish), the previously designed real-time PCR by Balcazar et al. (2007) was tested on spleen, kidney, intestine, gill and brain tissues from 40 rainbow trout (n=200), sampled from three Danish freshwater farms and one seawater farm where no signs of disease were observed and one seawater farm after two furunculosis outbreaks. Prevalence of *A. salmonicida* obtained by the real-time PCR was compared to the one obtained by bacterial culturing. The real-time PCR was chosen primarily due to the bulk of available previous research that have used the target and the fact that the plasmid pAsal1 and its derivatives are ColE2-type replicons, meaning they are high copy number plasmids (Lilly and Camps, 2015) making the assay highly sensitive. Moreover, all 20 Danish *A. salmonicida* that were tested were amplified by the assay. The real-time PCR showed a better result than culturing (65% vs. 30% positive fish by real-time PCR and culturing, respectively). The assay also indicated possible presence of carrier fish harbouring VBNC *A. salmonicida* in Danish fresh- and seawater rainbow trout farms and that the spleen, brain and intestine, where the bacterium was detected by real-time PCR and not culturing in fish showing no signs of disease, could play an important role in *A. salmonicida* infection and persistence of VBNC. This means that in order to detect possible carriers, one should strive for testing several tissues from fish instead of just one (usually the kidney). Moreover, sensitivity of the assay was 1 - 2 genomic units per reaction and the real-time PCR had a high reproducibility and an excellent efficiency, thus providing a sensitive tool for detection of *A. salmonicida.* However, the major drawback of the assay, which was exemplified by our later findings in Manuscript III by WGS of 99 *A. salmonicida*, is that the target plasmid is absent in some *A. salmonicida* and seemingly half

of the representative Danish *A. salmonicida* population and the absence does not necessarily have to be associated with lack of virulence nor culturing above 22 - 25^{o}C as indicated in earlier studies. In order to be certain of avoiding false negatives, another sensitive detection method with a different target would therefore need to be employed.

Chapter 4: Epidemiology

4.1 Epidemiology and genetic variation

One major factor for establishing an effective prevention strategy, besides enabling effective treatment, is to determine the epidemiology and genetic variation of the Danish *A. salmonicida*. Numerous molecular techniques have been applied for deciphering the epidemiology and genetic variation of *A. salmonicida* and generally the results support the notion of *A. salmonicida* being a highly homogenous subspecies as previously indicated by its phenotypical characteristics (Toranzo et al., 1991; Dalsgaard et al., 1994; Nielsen et al., 1994a).

One of the first studies using a molecular method for investigating genetic similarity of *A. salmonicida* was Belland and Trust (1988). Eleven *A. salmonicida* isolated from different species of fish and geographical places around the world were subjected to a DNA:DNA reassociation technique using a radiolabeled probe and the resulting mean DNA sequence similarity was 97 ± 6.1% (Belland and Trust; 1988). Other molecular methods have included restriction endonuclease fingerprinting analysis (McCormick et al., 1990), randomly amplified DNA polymorphism fingerprinting analysis (RAPD) (Miyata et al., 1995; Inglis et al., 1996; O'hici et al., 2000), amplified fragment length polymorphism (AFLP) (Lund et al., 2003b), plasmid profiling (Nielsen et al., 1993; Sørum et al., 1993), and ribotyping (Nielsen et al., 1994b). Although Nielsen et al. (1994b) and O'hici et al. (2000) did find some clustering using ribotyping and RAPD respectively; it was not enough to enable use of these methods for epidemiological studies except for maybe certain local geographical areas.

Pulsed-field gel electrophoresis (PFGE) has for a long time been the 'gold standard' for typing bacterial isolates, however, even this method did not have enough resolution for studying epidemiology of *A. salmonicida* (Garcia et al., 2000; O'hici et al., 2000; Cunningham and Colquhoun, 2002; Beaz-Hidalgo *et al.,* 2008). Garcia et al. (2000) examined 132 isolates from Denmark, Norway, Scotland, Ireland, the Faroe Islands, France, Canada and the USA and although PFGE was reportedly more useful to differentiate *A. salmonicida* than ribotyping, only slight differences were found and overall results suggested that a single clone of *A. salmonicida* was responsible for most of the worldwide furunculosis outbreaks. This theory is supported by findings of Nash et al. (2006). In order to identify virulence genes and possible vaccine candidates, Nash et al. (2006) developed a microarray-based comparative genomic hybridization (M-CGH) technique based on virulence associated genes from the genome sequence of *A. salmonicida* strain A449 (Reith et al., 2008). Nash et al. (2006) compared *A. salmonicida* and other *Aeromonas* species isolated from various fish species and geographic locations and found no correlation between host or geographic origin and the M-CGH patterns and a relatively low number of divergent genes in the *A. salmonicida* strains.

4.2 MLST-v

Currently, one of the most widely used molecular methods for typing microbial isolates is multilocus sequence typing (MLST) due to its great sensitivity, specificity and ease of use and data exchange (Enright and Spratt, 1998; Maiden et al., 1998; Platonov et al., 2000; Maiden, 2006; Martino et al., 2011; Martinez-Murcia et al., 2011). MLST is based on identifying polymorphic sites within DNA sequence fragments of multiple housekeeping genes. For each gene fragment, every sequence that differs is given its own unique allele no matter if they differ at one or several nucleotide sites. Each isolate is then characterized based on the combination of the alleles from all the gene fragments, whereby they can each be assigned a specific allelic profile or sequence type (ST) number (Maiden et al., 1998).

In the present thesis a modified MLST based on eight housekeeping genes (*dnaJ, rpoD, groL, gyrB, metG, ppsA, gltA* and *recA*) in combination with four virulence associated genes (*aexT, eno, vapA* and *fstB*) (Table 1) was applied on a preliminary representative collection of 23 Danish *A. salmonicida* isolates, one Scottish strain and the *A. salmonicida* type strain NCIMB 1102 in order to study their genetic variation using the publicly available WGS *A. salmonicida* strain A449 as reference (Reith et al., 2008) (Table 2). This kind of modified MLST, usually called MLST-v, has been used in previous studies where the method has proven to have a higher discriminatory power than MLST and has revealed important genetic information regarding virulence associated genes (Zhang et al., 2004;Chen et al., 2007; Doijad et al., 2014; Tankouo-Sandjong et al., 2007). A detailed materials and methods section for the MLST-v used in this thesis is described in Chapter 5: Methodological considerations.

The MLST-v scheme with allelic profiles for all *A. salmonicida* isolates was created, however, only five unique STs were identified (Table 2). The pattern of evolutionary descent of *A. salmonicida* based on the STs was also analyzed using eBURST (http://eburst.mlst.net/3.asp). Analysis showed that all isolates belong to the same clonal complex (CC), since no isolate differed by more than a single allelic mismatch. Nineteen of the Danish isolates and the type strain NCIMB 1102 belonged to the primary founder ST (ST 1) (Fig. 4A), which is the ST with the most single-locus variants (SLVs) (http://eburst.mlst.net/3.asp). This was supported with a bootstrap confidence level of 88% (percentage based on 1000 replicates). A Bayesian phylogeny tree based on the concatenated sequences from *A. salmonicida* MLST-v was also constructed, displaying the high homogeneity of the *A. salmonicida* (Fig. 4B). In conclusion, all analysis showed that the MLST-v was not an adequate tool for studying the epidemiology and genetic variation of Danish *A. salmonicida*. The results support the high genetic homogeneity of *A. salmonicida* found in previous studies using other typing methods (e.g. Belland and Trust; 1988; Beaz-Hidalgo et al., 2008; Nash et al., 2006).

Table 1. Information about primers used for PCR amplification and sequencing of housekeeping and virulence associated genes for development of a MLST-v scheme. The following is depicted in table form left to right: Primer names for each gene, nucleotide sequence for each primer, brief description of the protein product that the respective genes encode, length of the PCR amplicon of each gene fragment in base pairs (bp), length of the target sequence located within the PCR product of each gene fragment that is used for the MLST-v scheme, annealing temperature for the respective primer pairs used during PCR amplification, and reference article for each primer including this thesis as reference if primers were designed during the present thesis.

Primer	Sequence (5'-3')	Gene product	Length of PCR amplicon (bp)	Length of target sequence (bp)	Annealing temperature	Reference
gyrB-F	CATGTCTACGAGCA GACCTA	DNA gyrase (type II topoisomerase), subunit B	926	682	54 ^{0}C	(Martinez-Murcia et al., 2011)
gyrB-R	CTCCACGTTCAGGA TCTTGCC					
rpoD-F	GAAGGCGAAATCG ACATCGC	RNA polymerase, sigma 70 (sigma D) factor	700	649	55 ^{0}C	(Martinez-Murcia et al., 2011)
rpoD-R	ATGCTCATGCGRCG GTTGAT					
groL-F	CAAGGAAGTTGCTT CCAAGG	Chaperonin GroEL	782	604	57 ^{0}C	(Martino et al., 2011)
groL-R	CATCGATGATGGTG GTGTTC					
dnaJ-F	CGAGATCAAGAAG GCGTACAAG	Chaperone Hsp 40, co-chaperone with DnaK	934	814	54 ^{0}C	(Martinez-Murcia et al., 2011)
dnaJ-R	CACCACCTTGCACA TCAGATC					
recA-F	GCTGGGTCAGATTG AAAAGC	Recombinase A	640	635	57 ^{0}C	(Martinez-Murcia et al., 2011)
recA-R	CTCGCCGTTATAGC TGTACC					
gltA-F	TTCCGTCTGCTCTC CAAGAT	Citrate synthase I	626	373	57 ^{0}C	(Martino et al., 2011)
gltA-R	TTCATGATGATGCC GGAGTA					
metG-F	TGGCAACTGATCCT CGTACA	Methionyl-tRNA synthetase	657	539	57 ^{0}C	(Martino et al., 2011)
metG-R	TCTTGTTGGCCATC TCTTCC					
ppsA-F	AGTCCAACGAGTA CGCCAA	Phosphoenolpyruvate synthase	619	502	61 ^{0}C	(Martino et al., 2011)
ppsA-R	CTCGGCCAGATAG AGCCAGGT					
aexT-F	TGCAGATTCAAGC AAACACC	ADP-ribosylating toxin	689	608	61 ^{0}C	This thesis
aexT-R	GCCAGCAACTTCTG CCTTTA					
eno-F	CGCCGACAACAAC GTCGACATC	Enolase	598	518	56 ^{0}C	(Martino et al., 2011)
eno-R	CTTGATGGCAGCCA GAGTTTCG					
vapA-F	CAACGGTTTCATTG TGTTGG	A-layer	630	556	56.5 ^{0}C	This thesis
vapA-R	TTGAAGGCAGAAA CATCACC					
fstB-F	GTTTCCCGCTTTTC CTTGA	Ferric siderophore receptor B	594	512	58 ^{0}C	This thesis
fstB-R	GAAGATGCTGCGTT TGCTC					

Table 2.Overview of *A. salmonicida* isolates used in the MLST-v and their respective sequence type (ST) and allelic profile. For *A. salmonicida* isolates that were also used for whole genome sequencing (WGS), the same name is used as in manuscript III: The Danish isolates have a black color (freshwater farms) or a blue color (seawater farms) and are labeled by region of origin followed by year of isolation, with abbreviations Nj = Northern Jutland, Mj = Central Jutland, Sd = Southern Denmark, Sj = Zealand. The two Danish isolates that were not included in WGS are named Denmark followed by their year of isolation. There are five STs. Alleles *gltA*, *metG* and *vapA* are the only three alleles that vary in their sequence composition in at least one base pair (bp) among the isolates.

***A. salmonicida* isolate**	**ST**	**Alleles**											
		dnaJ	***gltA***	***groL***	***gyrB***	***metG***	***ppsA***	***recA***	***rpoD***	***aexT***	***eno***	***vapA***	***fstB***
Denmark 1985	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Mj13 1987	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Mj16 2008	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sd3 1982	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sd6 (a) 2013	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Mj21 1993	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Mj5 1986	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sd1 2004	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sd2 1982	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sd2 1995	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj2 (a) 1993	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj2 (b) 1993	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj3 (b) 2009	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj5 1994	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj5 1995	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj6 (a) 1993	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj6 (a) 1996	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj6 (b) 1993	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Sj6 (b) 1996	**1**	1	1	1	1	1	1	1	1	1	1	1	1
NCIMB 1102 (Type strain 1962)	**1**	1	1	1	1	1	1	1	1	1	1	1	1
Denmark 2009	**2**	1	1	1	1	1	1	1	1	1	1	2	1
Mj18 1986	**2**	1	1	1	1	1	1	1	1	1	1	2	1
Sj4 1982	**2**	1	1	1	1	1	1	1	1	1	1	2	1
Sd6 (b) 2013	**3**	1	1	1	1	1	1	1	1	1	1	3	1
Scotland	**4**	1	2	1	1	1	1	1	1	1	1	1	1
Reference A449 (France 1975)	**5**	1	1	1	1	2	1	1	1	1	1	1	1

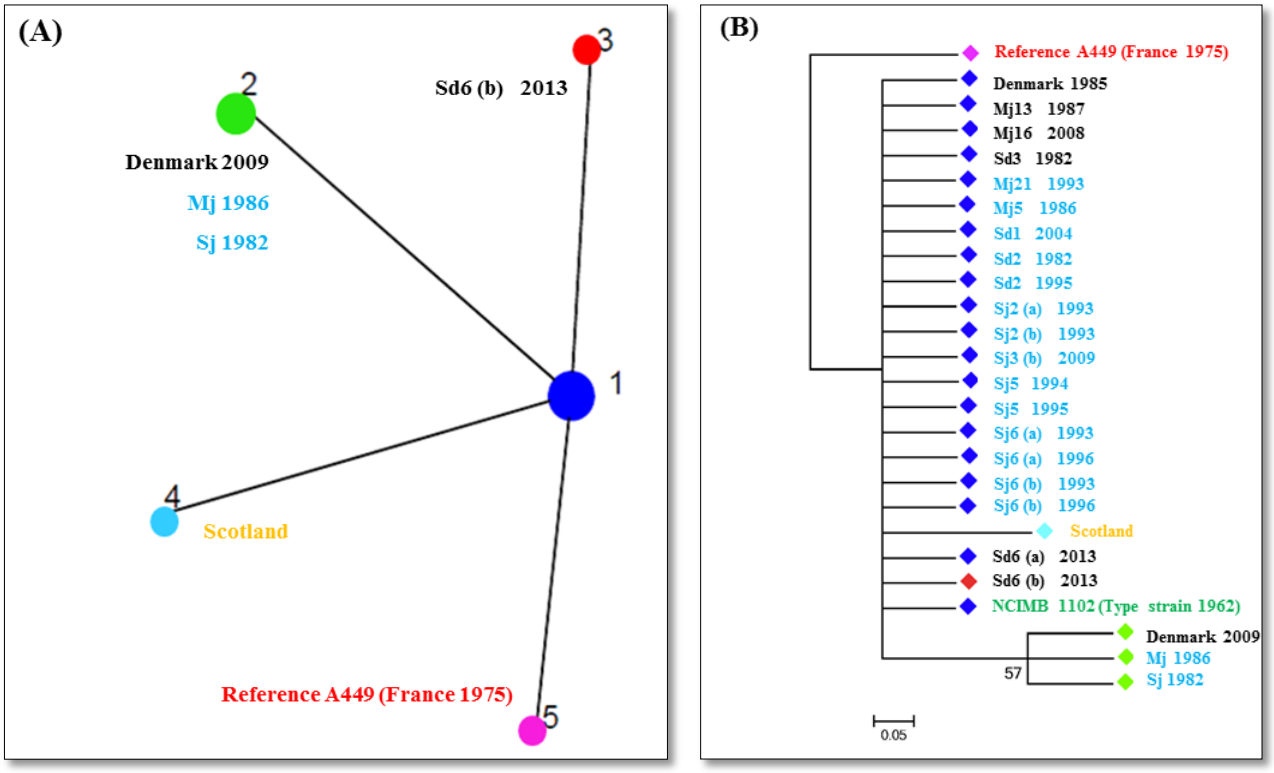

Figure 4. (A) Pattern of evolutionary descent of *A. salmonicida* STs from the MLST-v, as shown by eBURST. Nineteen of the Danish isolates along with the reference type NCIMB 1102 belong to ST 1. The three Danish isolates Denmark 2009, Mj18 1986 and Sj4 1982 belonged to ST 2, the Danish isolate Sd6 (b) 2013 belongs to ST 3, the Scottish isolate belongs to ST 4 and A449 belongs to ST 5. Same isolate labels are used as in Table 2. **(B)** Bayesian phylogeny tree based on the concatenated sequences from *A. salmonicida* MLST-v. The *A. salmonicida* isolates have a clonal population structure. The three Danish isolates belonging to ST 2 form a monophyletic group with a bootstrap value of 57%. Same isolate labels are used as in Table 2.

4.3 Whole genome sequencing

Previous molecular methods such as RAPD, AFLP, PFGE, MLST and MLST-v focus on a small part of the genome, while WGS can avoid such bias and thus provides the best overview for studying a population (Foxman et al., 2005; Parkhill and Wren, 2011). Since the first time bacterial genomes were completely sequenced in 1995 (Fleischmann et al., 1995; Fraser et al., 1995), WGS has gone through immense progress. The most notable factor for this progression was development and implementation of next-generation sequencing (NGS) techniques, initially introduced by Roche 454 Life Sciences in 2005 (Henson et al., 2012), which reduced the price and increased the speed of sequencing by reducing the average read length compared to sanger-sequencing (Rothberg and Leamon, 2008; Metzker, 2010; Land et al., 2015; Loman and Pallen, 2015). As a result, the number of sequenced genomes has skyrocketed in recent years (Land et al., 2015; Loman and Pallen, 2015). Although this has been accompanied by an increase in the recommended coverage needed for a genome assembly and number of contigs needing closure before a genome could be completed (Land et al., 2015), Illumina sequencing platform seems to be a cost-effective, since assemblies can be generated that are almost complete genomes (Mavromatis et al., 2012). Due to the relatively low costs along with its high accuracy, yield of error-free reads and percentage base calls above Q30

(Henson et al., 2012; Ross et al., 2013), the Illumina sequencing platform is currently the most widely used WGS technique (Metzker, 2010; Land et al., 2015).

NGS technology has also proved to be a powerful tool in microbiology for studying epidemiology of bacterial outbreaks (Hiller et al., 2007; Shendure and Ji, 2008; Pandya et al., 2009; Lewis et al., 2010; Leekitcharoenphon et al., 2012; Bertelli and Greub, 2013; Salipante et al., 2015; Stucki et al., 2015). The advantage with WGS lies with its high discriminatory power that enables deciphering the evolution of bacterial isolates belonging to the same clonal lineage, whereby highly robust phylogenies can be generated and the origin and routes of transmission of infections can be revealed (Parkhill and Wren, 2011). WGS even revealed the patient-to-patient pattern of transfer of a bacterial pathogen within a hospital (Harris et al., 2010). In the study by Salipante et al. (2015) PFGE was compared to WGS and although the result for relatedness of the bacteria by both methods correlated with each other, the resolution of WGS was highly superior. The same was concluded by Leekitcharoenphon et al. (2012) when investigating the epidemiology of *Salmonella enterica* using both PFGE and WGS. Leekitcharoenphon et al. (2012) moreover compared several WGS bioinformatics approaches for the same data and determined that nucleotide difference and single nucleotide polymorphism (SNP) tools were superior to the other bioinformatics methods. Indeed, the SNP approach has been frequently used for WGS based epidemiological studies (e.g. Pandya et al., 2009; Lewis et al., 2010; Stucki et al., 2015). In agreement, WGS in combination with the SNP analysis was able to distinguish among 101 sequenced *A. salmonicida*, using the published genome of the French strain A449 (Reith et al., 2008) as reference (Manuscript III).

Recently genome sequence information of numerous bacterial fish pathogens has also become available from genome sequencing projects, which is believed to become a vital part in finding new intervention strategies against bacterial infections in fish (Sudheesh et al., 2012). The genome of *A. salmonicida* isolate (A449) from a brown trout in the Eure river in France was sequenced by Reith et al. (2008). This has provided insight into the genomics of *A. salmonicida* that could prove to be vital for discerning the pathogen's evolution and infection progress (Reith et al., 2008). The study provided basic genetic information regarding the strains chromosome size of 4,702,402 base pairs (bp) and its plasmid profile consisting of two large plasmids pAsa4 and pAsa5 and three small plasmids pAsa1, pAsa2 and pAsa3. All of these plasmids, except for pAsa4, were also present among the majority of the *A. salmonicida* isolates sequenced in Manuscript III, where plasmids pAsal1 and pAsa6 were also additionally present in many isolates. Though, one of the key findings by Reith et al. (2008) included identification of functional genes encoded on plasmid pAsa5 and the chromosome for T3SS that has been shown to be vital for virulence in *A. salmonicida* as described in detail in chapter 1. Most likely due to rearrangements in pAsa5, both T3SS effector proteins and structural proteins were, however, absent in 24% of the isolates in Manuscript III. T3SS genes are also absent in the genome sequenced *A. hydrophila* ATCC 7966T and are only present on the chromosome in other *A. hydrophila* (Sha et al., 2005). The genome of A449 moreover contains numerous of virulence associated genes including the A-layer, toxins, secreted enzymes, iron acquisition genes, quorum sensing genes and ARGs (Reith et al., 2008) that were also found among the sequenced *A. salmonicida* in Manuscript III. Comparisons with the genome of *A. hydrophila* ATCC 7966T (Seshadri et al., 2006) also highlighted their respective evolution associated with possible adaptation to their fish hosts (Reith et al., 2008). Nevertheless, two Danish isolates from

brown trout, the French strain A449 also isolated from brown trout, one Scottish strain and the type strain NCIMB 1102 isolated from Atlantic salmon all showed high SNP similarity with the 97 Danish *A. salmonicida* isolated from rainbow trout in the WGS study of Manuscript III.

4.4 Manuscript III

Due to the lacking ability of MLST-v to distinguish among the homogeneous *A. salmonicida* isolates and the above mentioned advantages of WGS, we sequenced 99 Danish *A. salmonicida* isolated from different geographical regions and years (1980 - 2014), the Scottish strain MT004 and the type strain NCIMB 1102 from England. WGS was successfully able to distinguish among the *A. salmonicida* isolates, though it revealed that *A. salmonicida* is very homogenous, since only 667 SNPs were found among the isolates within the 4,702,402 bp long sequence of the chromosome. Bayesian temporal phylogenetic reconstruction showed that four major introductions of *A. salmonicida* into Denmark have occurred. The introductions correlate with the initial expansion of Danish rainbow trout production and the beginning of production in seawater. There is also a possibility that the bacterium might have initially been transmitted from seawater to freshwater. We moreover found some variation in plasmids and virulence factors, especially those encoded on plasmids and nine *A. salmonicida* harbored worldwide known ARGs against several antibiotics. Overall, our WGS analysis provided valuable information regarding epidemiology as well as genetic and virulence variations among the Danish disease causing *A. salmonicida* population.

Chapter 5: Methodological considerations

5.1 Materials and methods for MLST-v

Bacterial isolates

The following *A. salmonicida* isolates were selected for development a MLST-v scheme: 23 Danish *A. salmonicida* isolated between year 1982 and 2013 from various fresh- and seawater rainbow trout farms, one Scottish *A. salmonicida* isolated from Atlantic salmon and the type strain NCIMB 1102 isolated from Atlantic salmon (Table 2). The publicly available whole genome sequence of the French isolate A449 isolated from brown trout was used as a reference.

Primers

Primers for amplification of *A. salmonicida* gene fragments from eight housekeeping genes (*dnaJ, rpoD, groL, gyrB, metG, ppsA, gltA* and *recA*) and four virulence associated genes (*aexT, eno, vapA* and *fstB*) were either developed during the present thesis or obtained from previous publications (Martinez-Murcia et al., 2011; Martino et al., 2011). The list of housekeeping and virulence associated genes and all primers used for PCR amplifications and sequencing is listed in Table 1.

DNA extraction

All *A. salmonicida* were grown in VIB (Difco) for 48 h at 20°C. DNA was extracted using Qiagen QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol and stored at -20°C until further PCR amplification.

PCR reaction

PCR reaction was carried out in T3000 Thermocycler (Biometra) using a final volume of 50 µL containing: 5 µL GeneAmp® 10X Gold Buffer (150 mM Tris-HCl, pH 8.0, 500 mM KCl), 5 µL 25mM $MgCl_2$, 1 µL 10µM deoxyribonucleotide (dNTP) mix, 1 µL of each forward and reverse primer (l0 µM), 0.3 µL of AmpliTaq Gold® DNA Polymerase (5U μL^{-1}) (Applied Biosystems), 34.7 µL of sterile water and 2 µL of 5ng DNA genomic DNA as the template. The reaction mixture was subjected to denaturation at 94◦C for 3 min, followed by 35 cycles of amplification as follows: denaturation at 94◦C for 1 min, annealing temperature depending on the primer (table 2 and 3) for 1 min and extension at 72◦C for 1.30 min. At the end a final extension step at 72◦C was achieved for 3 min.

Sequencing

PCR Amplified products were analyzed by electrophoresis on 2% agarose E-gels (Invitrogen) and visualized on a UV transilluminator. The products were then purified using the High Pure PCR Product Purification kit (Roche Applied Science, Germany), following the manufacturer's instructions. Nucleotide sequences for the MLST-v analysis were determined by bidirectional

sequencing using the Big Dye Terminator V3.1 Ready Reaction Cycle Sequencing Kit in ABI 3130 Genetic Analyzer (Applied Biosystems, USA), according to the manufacturer's instructions. Each gene fragment was sequenced using the same forward and reverse primer pair as used for PCR amplification.

Data processing and analysis

Consensus sequences for twelve gene fragments from the *A. salmonicida* isolates used in the MLST were assembled via CLC Workbench software 6.5 (CLC Bio-Qiagen, Aarhus, Denmark). Alignment of all consensus sequences for each gene was done using the Clustal X program (Larkin et al., 2007). Trimming of alignments was done in Bioedit (Hall, 1999). All nucleotide variable positions and insertions/deletions found in the alignments were checked manually in raw sequence chromatograms by use of BioEdit (Hall, 1999) and CLC Workbench software 6.5. By use of nucleotide blast (Altschul *et al.,* 1990), all gene fragments (alleles) were found for the published isolate *A. salmonicida* A449 (Reith et al., 2008). Trimmed and edited sequences, including the *A. salmonicida* A449 sequences, were used to create a new MLST-v scheme for *A. salmonicida* in MLST plugin application in CLC workbench software 6.5 according to the program guidelines. Allele sequences that differed from each other by one or more polymorphisms were attributed to a unique allele number. Each unique allelic profile, as defined by the allele numbers of the twelve loci, was assigned a ST number. The same ST was assigned to isolates that shared the same allelic profile.

Analysis by eBURST

Pattern of evolutionary descent of 25 *A. salmonicida* isolates and the reference strain A449 was analyzed using eBURST (http://eburst.mlst.net/default.asp). The program uses allelic profiles to identify potential CCs and founders by linking SLVs or double-locus variants (Feil et al., 2004). Parameters for the analysis were set to stringent default setting, meaning all STs had to be SLVs of some other ST in the population, which is illustrated as a diagram with a single CC i.e. a group where all STs are linked (http://eburst.mlst.net/3.asp). Here the parameter was eleven, since twelve alleles (gene fragments) were used in the MLST-v.

Bayesian phylogenetic tree

Concatenated MLST-v sequences for 26 *A. salmonicida* isolates, including the reference strain A449, were subjected to Bayesian phylogenetic inference using Markov chain Monte Carlo (MCMC) methods in the software package MrBayes (Ronquist et al., 2012) available at: http://mrbayes.csit.fsu.edu/download.php. MCMC chains were simulated for one million generations with subsampling every 100 generations and an 'invgamma' molecular evolution model that allows a gamma distribution across positions of the alignment with invariable sites.

5.2 *In vivo* imaging (Manuscript I)

Before the experimental infection of rainbow trout of this study was carried out, the following was investigated: 1) the lowest possible immersion time of fish in the diluted *A. salmonicida gfplux*

culture that would enable attachment and thus visualization of the bacterium on the fish for the first time point and 2) the difference between luminescence emittance of infected and non-infected fish i.e. the fish autoluminescence. Eight rainbow trout were experimentally infected in three liters of 5×10^7 CFU ml^{-1} *A. salmonicida gfplux* culture in well-aerated 10 ± 1°C aquariums at our institute. Two fish were euthanized the following time points using an overdose of 3-aminobenzoic acid ethyl ester: 30 min, 1 hour, 2 hours and 3 hours post infection. All euthanized fish were washed twice in distilled water, dipped on a paper towel, and finally laid on a sterile Tissue Culture Dish (Greiner Bio-One, Germany). Fish were then transported to the Danish Cancer Institute in their respective culture dishes, where they were placed in the dark collection chamber of the IVIS spectrum imaging workstation (PerkinElmer) for bioluminescence image capture (30 s). One euthanized non-infected control fish was also scanned to compare autoluminescence emitted by non-infected fish with luminescence emitted from *A. salmonicida gfplux* infected fish. Luminescence emission from *A. salmonicida gfplux* was observed on all eight infected fish at each of the time points 30 min - 3 hours post challenge at one or several of the following sites: dorsal fin, pectoral fin, gills, oral and nasal cavity, and the eyes. While the attachment site patterns varied among individual fish, the most intensive luminescence emission was observed from fish infected for 2- and 3 hours respectively. Based upon this result, 2 hours of immersion time in *A. salmonicida gfplux* diluted culture was determined to be sufficient for the bacterium to attach to the fish and enable visualization of the bacterium in the IVIS. Measured autoluminescence from the non-infected control fish was scarce and could not be visualized, compared to the visualized intense luminescence emitted by the *A. salmonicida gfplux* from infected fish.

It would be recommended for future studies that an experimental infection set-up, similar as to the one described in Manuscript I, would be carried out. The differences would include fish being immersed for only 30 min and in lower concentrations of *A. salmonicida gfplux* in order to better mimic natural infections and the fish would be followed for a shorter and longer duration than 24 hours respectively, in order to allow visualization of the internal organs at various time points. Though, the details and optimal settings for the additional infection experiments would need to be investigated first. Only if the above mentioned experimental infections would be successfully employed, then one could also consider testing a co-habitation challenge using this method, which would be the most optimal way to mimic natural conditions of infection. The instability of the plasmid vector carrying the GFP and luciferase genes would also have been investigated more closely. One possibility would have been to do the transformation procedure of *A. salmonicida* over again in order to see if a more stable *A. salmonicida* transformant could be made. Another option could have been to try treating the fish with ampicillin before start of the experimental infection and thereby create a favorable environment inside the fish for *A. salmonicida gfplux* i.e. *A. salmonicida* carrying the plasmid vector with GFP, luciferase and ampicillin resistance.

5.3 Real-time PCR (Manuscript II)

Initially, several extraction methods for extracting *A. salmonicida* DNA from seeded tissue were tested, as well as different amounts of tissue in order to avoid inhibition of the real-time PCR assay. The three extraction methods that were tested extensively were Maxwell® 16 LEV Blood DNA Purification Kit (Promega), QIAamp DNA Mini Kit (Qiagen) and InstaGene Matrix (Bio-Rad). The

Maxwell and QIAmp kits are both standard kits at our institute and have been used for extraction from various tissues and fluids of fish and other animals with slightly modified versions of the manufacturers' procedures. However, even after exhaustive testing with various modifications e.g. overnight lysis, different buffer and proteinase K combinations and lowering the amount of all tissues to 10 mg, there were still problems with the extractions and especially the spleen. The main issue with the QIAamp DNA Mini Kit was that the filters seemed to become clogged by the tissue even after extended lysis procedures. Difficulties extracting with the Maxwell® 16 LEV Blood DNA Purification Kit included carryover of particles during the extraction process, lower than expected DNA yield and inhibition of real-time PCR. Briefly the Maxwell® 16 Tissue DNA Purification Kit was also tested, though the same problems occurred as with the previous Maxwell kit. The InstaGene Matrix kit, which has been used for extracting DNA from fish tissues in a number of previous PCR and real-time PCR studies e.g. (Balcazar et al., 2007; Keeling et al., 2012; Beaz-Hidalgo et al., 2013), gave the most optimal results and was thus used henceforth.

Two other previously developed real-time PCR assay by Goodwin and Merry (2009) and Keeling et al. (2012) respectively, were also tested before continuing with the assay developed by (Balcazar et al., 2007). The assay by Goodwin and Merry (2009) included three different PCR primers used for conventional PCR assays in the study by Byers et al. (2002b). One of the primers had the same target (plasmid pAsal1) as the real-time PCR primers of Balcazar et al. (2007), though the main difference was that Goodwin and Merry (2009) did not change the PCR primers even though they amplified long (421 - 512 bp) DNA sequences, which is a standard size for conventional PCR amplicons, though the recommended optimal amplicon size for real-time PCR is usually 50 - 200 bp. This might have contributed to the fact why we were not able to reproduce the results by Goodwin and Merry (2009), as the primers did not amplify pure cultures of various *A. salmonicida* strains. When testing the assay by Keeling et al. (2012) with primers targeting the *vapA* gene, similar results were obtained as with the assay by Goodwin and Merry (2009). The only assay that showed promising results was the one originally developed by Balcazar et al. (2007) and was thus pursued further.

Given the current knowledge obtained by WGS of the 99 Danish *A. salmonicida* isolates in Manuscript III, a different target gene than *aopP* encoded on pAsal1 would have been used for the real-time PCR assay. Due to limited amount of time, developing and implementing new primers was not possible. Nevertheless, if new primers were to be developed in the future, it would be essential to obtain the same high sensitivity as the one obtained in this real-time PCR assay.

5.4 Whole genome sequencing (Manuscript III)

In retrospect, due to the inability of the developed MLST-v to distinguish among the Danish *A. salmonicida*, the best solution would have been to avoid this method and instead have moved straight to WGS. Plasmid profiling of the 101 sequenced *A. salmonicida* isolates would also be recommended in the future for enabling a better comparison with previous studies of *A. salmonicida* plasmids.

Chapter 6: Discussion and future perspectives

6.1 Discussion

From a broader perspective, research conducted within the framework of this PhD project aimed at contributing to ongoing efforts towards resolving the current concerns of furunculosis in Danish rainbow trout production. As discussed in the following paragraphs, investigations of three key matters regarding this grave fish disease, done throughout this thesis, have contributed novel insights into each of these subjects.

The route of entry and dissemination of *A. salmonicida* was investigated by tracking the bacterium *in vivo* using BLI in Manuscript I. Although uncertainties regarding this topic remain, the *in vivo* study shed light on the initial host-pathogen relationship between rainbow trout and *A. salmonicida*. The key findings were that *A. salmonicida* mostly seemed to initially colonize the gills and the dorsal and pectoral fin and move on quite rapidly (within 24 hours) to internal organs such as the intestine, spleen and kidney. These findings, although more elaborate studies are needed to establish any firm conclusions, are in agreement with several previous studies regarding *A. salmonicida* (e.g. Willumsen, 1990; Hiney et al., 1997; Svendsen et al., 1999) and moreover highlight some key issues that need to be resolved in order to comprehend the initial stages of the *A. salmonicida* infections.

One of the important issues, stressed by the fact that the skin was not one of the suggested primary colonization sites of *A. salmonicida* in this study, was that fish species seem to have different levels of resistance to *A. salmonicida* that are correlated with the immune system and especially the mucosal activity of the species (McCarthy, 1977; Cipriano et al., 1994a; Dickerson, 2009). More attention should be paid to this important topic, especially since some studies have argued that the mucus of skin could be the best sampling site for carrier fish (Svendsen and Bøgwald, 1997). This might be true for more susceptible fish species like Atlantic salmon (McCarthy, 1977), but does not necessarily apply to rainbow trout due to their high resistance and the indicated lack of skin colonization observed in Manuscript I. Granted, the results from the *in vivo* study cannot be compared to carrier infections due to the high amount of bacteria used (5×10^7 CFU ml^{-1} *A. salmonicida gfplux* culture) for the immersion and non-natural infection conditions, nevertheless, it emphasizes that progression of *A. salmonicida* in fish is not uniform in all species, meaning one cannot rely on certain detection and prevention strategies for *A. salmonicida* being applicable for all farmed fish if only certain species have been investigated.

Another notable result in Manuscript I was the fast dissemination of *A. salmonicida* into the internal organs of the fish. Again it is important to emphasize that the fish were immersed in a high concentration of the bacterium, which could have contributed to this rapid dissemination. Nonetheless, it is an intriguing observation supported by other studies concerning digestion of various feed (e.g. Windell *et al.* 1969) that one should bear in mind in future research regarding this bacterium. Returning to the subject of carrier fish, this rapid dissemination pattern might also provide insight into possible colonization sites of the bacterium in carrier fish. If the pathogen is only attached to the outer surface of the fish for a short period of time, at least concerning rainbow trout,

one could thus speculate whether the focus should be shifted towards sampling of internal organs. This suggestion is supported by the real-time PCR findings of Manuscripts II, where *A. salmonicida* in 20 rainbow trout without signs of disease was found only in internal organs (the brain, spleen and intestine) and not in the gills. These findings also highlight the importance of sampling from more than one organ in order to detect *A. salmonicida*, whereby we move on to the second key subject of this thesis, namely development of a highly sensitive method for detection of the bacterium. On one hand this was accomplished, since the real-time PCR enabled detection of 1 - 2 genomic units per reaction and showed more sensitive detection of *A. salmonicida* than the culturing method. The difference in sensitivity was even more profound in fish without signs of disease (possible carriers), where only one out of 20 fish was found positive by culturing, while six fish were positive with real-time PCR. The major drawback of the real-time PCR, limiting the future applicability of this method for future field investigations in at least Denmark, is the fact that 50% of the representative *A. salmonicida* population in Denmark was missing the target sequence of the assay (Manuscript III). Previous studies have shown that the absence of the target (plasmid pAsal1) can be caused by culturing conditions above 22 - 25^{o}C (Daher et al., 2011) and it has been argued that the absence could be related to lack of virulence of the bacterium (Balcazar et al., 2007). However, it was revealed by WGS in Manuscript III that these two conditions are not necessarily associated with the absence of pAsal1, since all the *A. salmonicida* isolates were cultured at 20^{o}C and were isolated from furunculosis outbreaks.

One should nevertheless, keep in mind the two key factors that were highlighted through the findings of Manuscript II, which were the importance of sampling from multiple organs when trying to detect *A. salmonicida* and the indication of possible carriers being present in Danish freshwater farms. The most noteworthy aspects regarding the topic of sampling sites for *A. salmonicida*, is that the intestine might be a colonization site for *A. salmonicida* as indicated by findings in Manuscript I and that the brain could also be a possible colonization site. Both organs, along with the spleen, were the only positive organs in potential carrier fish, while the intestine is rarely used for sampling and to the best of our knowledge the brain has never been investigated in any previous studies of *A. salmonicida.* The indication of possible presence of carrier fish in Danish freshwater farms supports the widespread theory of freshwater to seawater (via carrier fish) *A. salmonicida* transmission in Denmark. In congruence, this transmission pattern of *A. salmonicida* was seen in Manuscript III for isolates harboring ARGs. Nevertheless, the four major introductions of *A. salmonicida* into Denmark that correlate with the initial expansion of Danish rainbow trout production and the beginning of production in seawater, indicated the possibility of the bacterium initially being transmitted from seawater to freshwater (Manuscript III). This brings us to the last key investigation of this thesis, namely obtaining knowledge on the epidemiology and genetic and virulence variation of the Danish disease causing *A. salmonicida*.

Although the Bayesian temporal phylogenetic reconstruction based on the SNPs obtained from WGS gave a general insight into the epidemiology of the Danish *A. salmonicida* population, it was difficult to find specific geographical correlations between the local fish farms, which might have been caused by a trade of fish for anglers and between individual farms. While WGS also proved to be a valuable tool for distinguishing among the homogenous Danish *A. salmonicida* isolates, overall it still supported the notion of high homogeneity, since only 667 SNPs were found among all 101

isolates (two from other countries). This finding also explained why almost all *A. salmonicida* used in the initially developed MLST-v had identical sequences.

Variation of *A. salmonicida* seemed mainly to be related to the plasmid profiles and virulence factors encoded on these plasmids, of which T3SS related virulence proteins seemed to be the most variable ones. This was associated with the high instability of pAsal1 and pAsa5 on which most of the T3SS virulence proteins are encoded. Though, *A. salmonicida* can harbor plasmids that share the same virulence genes e.g. plasmid pAsa6 that was included in the study (Manuscript III) and that harbor insertion sequences that can cause rearrangements and enable possible transfer of genes between plasmids and the chromosome (Daher et al., 2011; Dallaire-Dufresne et al., 2014). This intertwined system of virulence-associated genes makes is difficult to state anything concrete regarding this topic, without a more comprehensive analysis of the T3SS proteins. What can be suggested from the findings of Manuscript III regarding T3SS and the previously shown significance of its structural genes for *A. salmonicida* virulence (Burr et al., 2002; Burr et al., 2003; Stuber et al., 2003; Burr et al., 2005; Dacanay et al., 2006; Froquet et al., 2007), is that this theory needs to be investigated more thoroughly because the sequences encoding some of the structural proteins (e.g. *ascV*) were absent in 24% of the 101 sequenced *A. salmonicida*. The isolates that were missing these structural proteins have not been subjected to any *in vivo* studies that could provide information regarding their virulence or possible lack thereof, however, these *A. salmonicida* were isolated from furunculosis outbreaks. One can speculate whether this group of isolates could have lost these protein-coding sequences during culturing, since stressful culture conditions can cause rearrangements in plasmid pAsa5 whereby T3SS related genes can be lost (Stuber et al., 2003; Tanaka et al., 2012; Dallaire-Dufresne et al., 2014). On the contrary to this theory, all the Danish *A. salmonicida* were cultured at 20^{o}C.

Another significant finding was that nine *A. salmonicida* harbor worldwide known ARGs against several antibiotics, among these were ARGs against trimethoprim and sulphonamide that are licensed antibiotics for treatment in Danish aquaculture. The prevalence of these ARGs was low (9%), nevertheless, there is a clear transmission pattern of some of the isolates harboring the ARGs from a freshwater farm to several seawater farms. The ARGs found in the Danish isolates have also been found in *Aeromonas* species isolated from different environments around the world (L'Abée-Lund and Sørum, 2001; Sørum et al., 2003; Kadlec et al., 2011; Muziasari et al., 2014), supporting the evidence of the widespread dissemination of ARGs. This also highlights the need for developing an effective vaccine. Based on the findings of Manuscript III, the A-layer protein and GCAT protein sequences seem to be highly homogeneous among the Danish *A. salmonicida*, which warrants further investigation into their potential use for a vaccine against *A. salmonicida*. On the other hand, further studies are needed to identify any specific isolate(s) and/or virulence factor(s) that could be recommended in implementation of a vaccine.

6.2 Conclusion and future perspectives

The bacterium *A. salmonicida* was discovered over 100 years ago, however, there are still many questions regarding this pathogen and the disease furunculosis that remain unanswered. The findings

of this project have provided some valuable information for research on *A. salmonicida*, however, there is much that could be improved and further elaborated.

The best way to gain more knowledge on the host-pathogen relationship of *A. salmonicida* is through *in vivo* imaging, a valuable approach that is rapidly advancing. Although fluorescent and bioluminescent reporters are still the most frequently used reporters for imaging, their limitations of resolution and range of depth prevent the possibility of obtaining a detailed picture of the host-pathogen relationship. Notably, one emerging method that promises to revolutionize imaging and surpasses both of the above methods in resolution and range of depth is photoacoustic imaging, which uses ultrasound waves for imaging (Xu and Wang, 2006). Thus far this technology has only been used for human biomedical research; however, in the near future this imaging method could become available in the veterinary field.

Another detection method for *A. salmonicida* that would have the same high sensitivity as the real-time PCR assay in Manuscript II, but would enable 100% detection of all *A. salmonicida*, could be developed by changing the target of the present assay. One possible target could be the high-copy number plasmid pAsa1 that thus far seems to be universally present and stable in all *A. salmonicida* (Attéré et al., 2015).

Much data has been obtained by WGS of the 101 *A. salmonicida* isolates and only a fraction of this data has been utilized for analysis thus far. Indeed, much more valuable and in-depth knowledge could be found by applying some of the available bioinformatics tools, a promising goal for which the dataset created by our WGS analyses can provide a solid foundation.

Taking all the obtained results throughout this thesis into consideration, this PhD project has contributed novel and insightful information further promoting the current research on Danish disease causing *A. salmonicida* as well as the bacterium in general. There still seems to be a challenging 'road ahead' when trying to prevent furunculosis in the Danish rainbow trout production. On the positive side, one can hope that the findings of this project will inspire future research on *A. salmonicida* and that eventually prevention of furunculosis will be successful before even greater losses are seen due to the constant increase in intensity of farming combined with the major disease-promoting environmental stress factor – the overall raising global temperature.

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Accompanying manuscripts I - III

Manuscript I

Infection routes of *Aeromonas salmonicida* in rainbow trout monitored *in vivo* by real-time bioluminescence imaging

Infection routes of *Aeromonas salmonicida* in rainbow trout monitored *in vivo* by real-time bioluminescence imaging

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Abstract

Recent development of imaging tools has facilitated studies of pathogen infections *in vivo* in real time. This trend can be exemplified by advances in bioluminescence imaging (BLI), an approach that helps to visualize dissemination of pathogens within the same animal over several time points. Here we employ bacterial BLI for examining routes of entry and spread of *Aeromonas salmonicida* susbp. *salmonicida* in rainbow trout. A virulent Danish *A. salmonicida* strain was tagged with pAK*gfplux*1, a dual-labeled plasmid vector containing the mutated *gfpmut3a* gene from *Aequorea victoria* and the *luxCDABE* genes from the bacterium *Photorhabdus luminescens*. The resulting *A. salmonicida* transformant exhibited growth properties and virulence identical to the wild type *A. salmonicida*, which made it suitable for an experimental infection, mimicking natural conditions. Fish were infected with pAK*gfplux*1 tagged *A. salmonicida* via immersion bath. Colonization and subsequent tissue dissemination was followed over a 24-hour period using the IVIS Spectrum imaging workstation. Results suggest the pathogen's colonization sites are the dorsal and pectoral fin and the gills, followed by a progression through the internal organs and an ensuing exit via the anal opening. The present work provides a tool for visualizing colonization of *A. salmonicida* and other bacterial pathogens in fish.

Introduction

Aeromonas salmonicida susbp. salmonicida, the causative agent of the disease furunculosis, is one of the major bacterial pathogens in aquaculture throughout the world. Furunculosis is a septicemic infection that was first described in Denmark in freshwater rainbow trout (*Oncorhynchus mykiss*) farms during the 1950s (Rasmussen 1964). Currently, furunculosis causes the greatest problems in

Danish rainbow trout production in sea water, where outbreaks occur during stress-associated periods with elevated temperatures around 20^{o}C in July and August (Larsen & Mellergaard 1981; Dalsgaard & Madsen 2000; Pedersen, Skall, Lassen-Nielsen, Nielsen, Henriksen & Olesen 2008). This indicates a possibility of *A. salmonicida* being spread from freshwater into the sea via rainbow trout carriers (Dalsgaard & Madsen, 2000). However, attempts to isolate bacteria from these presumed carrier fish have not been successful so far (Dalsgaard & Madsen 2000). Consequently, discerning the sites of entry and dissemination pattern of *A. salmonicida* within fish could be an important factor for optimizing future sampling procedures and detection of the bacterium.

Given the advances in the field of *in vivo* imaging in recent years, real-time monitoring of pathogens with various fluorescence and luciferase protein reporters has emerged as a promising strategy for following the pathogens' dissemination within their hosts (Contag, Contag, Mullins, Spilman, Stevenson & Benaron 1995; Rocchetta, Boylan, Foley, Iversen, Letourneau, McMillian, Contag, Jenkins & Parr 2001; Karsi, Menanteau-Ledouble & Lawrence 2006; Karsi & Lawrence 2007; Zinn, Chaudhuri, Szafran, O´Quinn, Weaver, Dugger, Lamar, Kesterson, Wang & Frank 2008; Menanteau-Ledouble, Karsi & Lawrence 2011). In comparison to fluorescence reporters, luciferase proteins that exhibit bioluminescence (BLI) show higher sensitivity, lower toxicity and faster response to changing environments (Burns, Joh, Francis, Shortliffe, Gruber, Contag & Contag 2001; Troy, Jekic-McMullen, Sambucetti & Rice 2004). Moreover, there is scarcely any autoluminescence (BLI background noise) emitted by animal tissues, as opposed to other light sources where the background disturbance emitted by tissues usually affects the light-emitting reporters' sensitivity and overall applicability (Troy *et al.* 2004).

BLI has been applied for monitoring *A. salmonicida* in dead Atlantic salmon (*Salmo salar*) (Ferguson, Bricknell, Glover, MacGregor & Prosser 1998). However, requirement for an exogenous addition of aldehyde for BLI visualization and incorporation of luciferase genes into *A. salmonicida* significantly lowered the virulence of the bacterium (Ferguson *et al.* 1998). First application of BLI for real-time monitoring of bacteria in live fish was reported by Karsi *et al.* (2006) who used the method to investigate dissemination of *Edwardsiella ictaluri* inside channel catfish (*Ictalurus punctatus*). Recently, Méndez & Guijarro (2013) used BLI to trace dissemination of *Yersinia ruckeri* in rainbow trout. Both studies used a bacterial luciferase operon from *Photorhabdus luminescens* consisting of five genes (*luxCDABE*) encoding the luciferase and fatty acid reductase enzyme complex, enabling emission of luminescence without the addition of any cofactors or exogenous substrates (Meighen 1993; Burns *et al.* 2001; Troy *et al.* 2004).

The aim of this study was to explore the routes of entry and subsequent tissue dissemination of *A. salmonicida* by using *in vivo* imaging and luciferase coding operon (Karsi *et al.* 2006; Méndez & Guijarro 2013). In short, a virulent *A. salmonicida* was tagged with a dual-labeled reporter plasmid containing a mutated green fluorescence protein (GFP) gene and the BLI coding genes (*luxCDABE*) under the same *lac*Z promoter that requires only oxygen for constitutive expression of both protein reporters (Karsi & Lawrence 2007). Expression of GFP provided a marker for verifying the presence of plasmid in the tagged bacterium and proper function of the *lac*Z promoter, while expression of BLI provided a marker for *in vivo* monitoring of a bacterium in the host after experimental infection.

Materials and Methods

Bacterial strains and plasmid transfer

Aeromonas salmonicida subsp. *salmonicida* 090710-1/23 (further referred to as *A. salmonicida* WT) is a virulent strain from a disease outbreak in Denmark. This strain has been used several times for various infection experiments in our and other Danish laboratories (e.g. Chettri, Skov, Jaafar, Krossøy, Kania, Dalsgaard & Buchmann 2015). *Escherichia coli* B/K 12 (Addgene, USA) is a donor strain that has an incorporated plasmid vector pAK*gfplux*1 consisting of the plasmid pBBR1MCS4 with an inserted: 1) *gfpmut3a* mutant gene from *Aequorea Victoria,* 2) *luxCDABE* operon from *Photorhabdus luminescens*, and 3) an ampicillin resistance gene. The pAK*gfplux*1 plasmid was first isolated from the *E. coli* B/K 12 donor strain by QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), according to the manufactures instructions. The plasmid was then transformed into *A. salmonicida* WT by conjugal mating using a natural kanamycin resistant *E. coli* strain SM10 λpir (Biomedal, Spain) as described by Karsi *et al.* (2007).

In details, natural kanamycin resistant SM10 λpir was transformed with pAK*gfplux*1 plasmid carrying ampicillin resistance by electroporation using a Gene Pulser instrument (Bio Rad) at 25 µFD, 200 Ω, 1.8 kV and with a time constant (tau value) of 5 msec. SM10 λpir ampicillin and kanamycin resistant colonies were grown overnight in 2 ml of Luria Bertani (LB) medium (Difco) at 37^{o}C with shaking at 225 rpm. The recipient was grown separately for 48 h in Veal Infusion Broth (VIB) (Difco) at 20^{o}C. Subsequently, 750 µl of the donor *E. coli* SM10 λpir and 1.5 ml of recipient culture were centrifuged separately at 8700 g for 2 minutes and the supernatant was removed. Collected bacteria were then washed by resuspending the pellets in 750 µl of Brain Heart Infusion (BHI) (Difco). Washing procedure was repeated three times in order to remove antibiotics used during broth culture. In 1.5 ml centrifuge tubes, 50 µl of donor and 100 µl of recipient washed

cells were mixed and bacteria were centrifuged as previously. Supernatant was poured off and donor and host mixture was suspended in 5-10 µl of BHI. Punched and sterilized 0.45 µM filters (Life GE Healthcare Life Sciences) were placed on blood agar plates (Columbia agar base (Oxoid) with 5% calf blood) without antibiotics and 5 µl of bacterial mixture was transferred on the filter as a spot. Plates were returned to incubator set to suitable temperature for the recipient (20^{o}C) and conjugation continued for 48 h. At the end of the conjugation period, filters were dropped into sterile 1.5 ml Eppendorf tubes and bacteria were washed away by adding 750 µl of VIB with 100µg ml^{-1} ampicillin (Sigma-Aldrich). Ten microliters of bacterial suspension were mixed with 990 µl of ddH_2O and 25 µl of the mixture was spread onto selective blood agar plates containing 100 µg ml^{-1} ampicillin and incubated for 48 h at 20^{o}C. Incubation temperature 20^{o}C ensured optimal growth of *A. salmonicida* and ampicillin selected for *A. salmonicida* with incorporated pAK*gfplux*1 (further referred to as *A. salmonicida gfplux*). *A. salmonicida gfplux* colonies showing the strongest fluorescence under an Axio imager M1 (Zeiss, Germany), were transferred into 5 ml VIB and incubated for 48 h at 20^{o}C. The bacteria were subcultivated two times under these conditions and then two times using blood agar plates without ampicillin, from which a single *A. salmonicida gfplux* colony was selected for storage in glycerol stocks (600 µL of 50% glycerol with 900 µL of the 48 h grown bacterial culture in VIB) at -80^{o}C until further use.

Experimental fish

Fertilized eggs of rainbow trout from Fousing Trout Farm (Jutland, Denmark) were brought to our institute, where disinfection, hatching and rearing were carried out under pathogen-free conditions. Fish were held at 10 ± 1°C in 180-liter tanks containing a flow-through system with non-chlorinated tap water and air supply. Fish were fed dry commercial feed (Inicio Plus; BioMar A/S, Denmark) at

1% of biomass per day. Average weight and length of rainbow trout used for all challenges was 8.8 ± 2.7 g and 9.3 ± 1.7 cm. Experimental infections were carried out in accordance with the accepted guidelines for the care and use of laboratory animals in research and with regulations set forward by the Danish Ministry of Justice and Animal Protection committees by Danish Animal Experiments Inspectorate permit number 2013-15-2934-00976.

Sensitivity of IVIS for detecting *A. salmonicida gfplux*

To determine the threshold detection limit for visualization of *A. salmonicida gfplux* using an IVIS spectrum imaging workstation (PerkinElmer) with an exposure time of 30 s, four separate two-fold serial dilutions in 0.9% saline solution ranging from 8×10^4 - 2×10^2 colony-forming-units (CFU) ml^{-1} of the bacterium grown in VIB for 48 h at 20°C, were made in a black 96-well microtiter plate (Thermo Scientific) using a volume of 0.1 ml per well. To measure background noise (autoluminescence), aliquots of 0.1 ml 0.9% saline solution were also added to four wells as controls. The plate was scanned for 30 s. Relative intensity of luminescence emission for each well was estimated by IVIS software and represented with a pseudo-colour scale of counts s^{-1}. Each dilution series was then cultivated on blood agar plates to confirm CFU ml^{-1} and after 48 h growth at 20°C, all plates were visualized using an Axio imager M1 in order to observe fluorescence emitted from the bacterial colonies. Mean autoluminescence was subtracted from luminescence values of all *A. salmonicida gfplux* wells and correlation between CFU ml^{-1} and relative intensity of luminescence emission was determined.

Plasmid stability and effect of transformation on bacterial growth *in vitro*

158 Plasmid stability was previously investigated by Karsi *et al.* (2006) and Karsi and Lawrence (2007).
159 Results from these studies suggested that the broad host range vector employed in this study can be
160 transferred and stably maintained in Gram-negative bacteria.
161
162 In order to test whether the introduction of plasmid affected growth properties of the wild-type
163 strain, bacterial growth of *A. salmonicida* WT and *A. salmonicida gfplux* were compared: 1)
164 indirectly using procedure by Karsi *et al.* (2006) for measuring optical density of bacterial culture
165 and 2) directly by plating serial dilutions onto blood agar to obtain CFU ml^{-1}. Readings of OD_{625}
166 were analyzed by a Student's paired t test using the Microsoft Excel statistical package. After the 48
167 h period, a serial dilution was made from each bacterial culture and plated on blood agar to
168 determine CFU ml^{-1} and fluorescence emitted from the bacterial colonies was observed using an
169 Axio imager M1. Determined CFU ml^{-1} was compared by a Student's t test analysis using Microsoft
170 Excel. The experiment was repeated three times.
171
172 **Effect of transformation on *in vivo* virulence**
173
174 *In vivo* virulence of *A. salmonicida* WT and *A. salmonicida gfplux* was compared by determination
175 of CFU required to infect 50% of the fish population. Bacteria were grown in VIB for 48 h at 20^{o}C.
176 Subsequently, ten-fold serial dilutions of bacteria, containing 10^{8} - 10^{4} CFU ml^{-1} were made in 0.9%
177 saline solution. Each dilution was then used for inoculation of six fish. Each fish was inoculated
178 intraperitoneally with 0.1 ml of the corresponding dilution, so the infection doses administrated
179 ranged from 10^{7} - 10^{3} CFU. One control group with six fish was injected with 0.1ml sterile veal
180 infusion broth, and one control group with six fish was left uninjected. Fish were held at 10 ± 1°C
181 in 10-liter tanks containing a flow-through system with non-chlorinated tap water and air supply.

Fish were observed several times per day and moribund fish were euthanized by immersion in 250 mg l^{-1} of 3-aminobenzoic acid ethyl ester (MS-222) (Sigma-Aldrich) until it was certain that swimming and gill movement had ceased. After two weeks the experiment was terminated. In order to confirm bacterial infection and to estimate plasmid stability during infection of the host, spleen, kidney and brain specimens were cultivated on blood agar and LB agar containing 100 µg ml^{-1} ampicillin. Fluorescence emission in the isolated bacteria was observed by using an Axio imager M1. Calculation of ID50 was done according to Reed & Muench (1938) and Student's t test in Microsoft Excel was used to assess significance.

Experimental setup for real-time monitoring *A. salmonicida gfplux in vivo*

Two fish were experimentally infected with *A. salmonicida gfplux* by separate immersion in two well-aerated 19 ± 1°C 10-liter tanks containing three liters of 5×10^7 CFU ml^{-1} *A. salmonicida gfplux* for two hours. For visualization each fish was anesthetized with MS-222, immersed twice in distilled water and dipped on a paper towel before finally being laid on a Tissue Culture Dish (Greiner Bio-One, Germany) and scanned in the IVIS for 30 s to estimate relative intensity of luminescence emission (counts s^{-1}). After scanning, each fish was placed in a new well-aerated 10-liter tank containing three liters of distilled water for recovery. Visualization was performed at the following time points: 2, 4, 6 and 24 h after immersion in the infection bath. Fish were euthanized before the last visualization time-point (24 h). Scanning in the IVIS was done on the whole fish and for the last scanning also on fish that were cut open to expose internal organs. Finally, for bacteriology examination, kidney and spleen samples were taken from each fish and streaked on blood agar that was incubated for 48 h at 20°C. Subsequently all plates were examined in an Axio imager M1microscope for fluorescence emission. One non-infected (control) fish was also scanned

in the IVIS as whole and cut open for monitoring of autoluminescence (counts s^{-1}) emitted from different external as well as internal areas. Obtained autoluminescence was subtracted from all luminescence readings of infected fish in order to acquire the correct luminescence (counts s^{-1}) for each scanning, which could then be correlated to a CFU count and be represented with a pseudo-colour scale. The experiment was repeated three times using one fish per tank and two times using two fish per tank. A total of fourteen infected fish and five non-infected control fish were examined.

Results

Detection limit of *A. salmonicida gfplux*

Visualization and measurement of the *A. salmonicida gfplux* two-fold dilutions showed that for the exposure time of 30 s, the threshold detection limit for visualization of *A. salmonicida gfplux* appears to be 4×10^4 CFU ml^{-1}. Correlation between CFU ml^{-1} and measured relative intensity of luminescence emissions was linear ($R^2 = 0.977$) over the range of 8×10^4 - 2×10^2 CFU ml^{-1} (Fig. 1), indicating luminescence emission should present the accurate CFU values for a given sample. Average percent of *A. salmonicida gfplux* colonies emitting fluorescence on a blood agar plate was 95% (data not shown).

[Figure 1]

Effect of transformation on bacterial growth *in vitro*

No significant difference was observed between *A. salmonicida* WT and *A. salmonicida gfplux* growth as measured by the indirect method of OD_{625} when analysed with a Student's paired t test ($p > 0.05$). Bacterial CFU ml^{-1} after 48 h of growth at $20^{o}C$ for each experiment was analysed by a Student's t test and showed no significant difference ($p > 0.05$) in all experimental repeats (Table 1). Average percent of *A. salmonicida gfplux* colonies emitting fluorescence on a blood agar plate in all experimental repeats was 96%, while no fluorescence emission was observed in any *A. salmonicida* WT colonies (data not shown).

[Table 1]

Effect of transformation on *in vivo* virulence

The ID50 value for *A. salmonicida* WT was 5×10^5 CFU and for *A. salmonicida gfplux* 6×10^5 CFU. Insertion of pAK*gfplux*1 into *A. salmonicida* WT did not seem to affect *in vivo* virulence of the bacterium. *A. salmonicida gfplux* colonies were re-isolated from all three sampled organs i.e. kidney, spleen and brain in euthanized fish. The number of colonies emitting fluorescence on blood agar plates gradually decreased over the course of the experiment and after 10 days fluorescence emission could not be detected any longer (Table 2). Colonies grown on LB agar with ampicillin showed consistently strong fluorescence but their number rapidly decreased. From day 5 of the experiment only few colonies grew on LB agar and after the day 8 no growth was recorded (Table 2).

[Table 2]

Experimental infection for real-time monitoring *A. salmonicida gfplux in vivo*

For the experimental infection, luminescence signal from *A. salmonicida gfplux* was observed in overall twelve of the fourteen examined fish following a two-hour immersion time (Table 3). At the 2-hour time point, a luminescence signal was detected on eight of the twelve positive fish. Bacteria were visualized on the following sites: the dorsal, pectoral, caudal and anal fin, anal opening, gills, oral and nasal cavity and eyes (Table 3; Fig. 2).

[Figure 2]

For the 4- and 6-hour time points, luminescence emission could be seen in three fish (Table 3). In two fish, the gills where observed luminescence was found at the 2-hour time point were still emitting luminescence at the 4-hour time point. Luminescence was also observed in the body organ area in one fish at the 6-hour time point, which was presumed to be emitted from the inside of the fish.

[Table 3]

After 24 hours, luminescence was found in four of the total of twelve positive fish (Table 3). Among the ten fish where the luminescent bacteria were found, in three fish the luminescence signal was strong enough to be seen through the skin in whole fish (Table 3; Fig. 3). In one of the three fish, the luminescence signal was located around the anal opening and after being cut open; the signal was still located around that area and the lower intestine (Fig. 3). The seven remaining fish needed to be cut open in order to be able to detect a luminescence signal. In all ten positive fish

277 at the 24-hour time point, the signal was located in the intestine (Table 3). In one fish the signal was
278 also located in the stomach (Table 3 and Fig. 3). In two fish the signal seemed to be located both in
279 the intestine and spleen. Bacteriological examination of kidney and spleen samples was positive for
280 *A. salmonicida gfplux* in all fourteen fish. However, very faint or no fluorescence emission was
281 observed from these colonies (data not shown). In all five non-infected control fish no bacteria were
282 isolated and measured autoluminescence emission was scarce compared to measured luminescence
283 emission from infected fish (negative data not shown).
284
285 **[Figure 3]**
286
287 **Discussion**
288
289 Experimental infections with *A. salmonicida gfplux* provided an indication of potentially important
290 colonization sites of *A. salmonicida*. However, colonization and dissemination of *A. salmonicida*
291 *gfplux* in fish could only be visualized in twelve of the fourteen experimentally infected fish and
292 after 24 hours bacteria were only visualized in the digestive system, while bacteria were re-isolated
293 from the spleen and kidney in all fourteen fish after 24 hours. The lack of visualization in other
294 organs could be due to the bacterial amount being below the threshold detection limit and/or a lack
295 of plasmid stability. The later scenario is supported by the fact that the re-isolated bacteria on blood
296 agar plates either emitted very little or no luminescence at all. Previous studies using plasmids with
297 the same luciferase coding operon as in this study did not report on any difficulties regarding
298 plasmid stability within fish (Karsi *et al.* 2006; Menanteau-Ledouble *et al.* 2011; Méndez &
299 Guijarro 2013). It is not possible to explain the reason(s) for the observed instability of the plasmid
300 within fish in this study.

When comparing the threshold detection limit of BLI to previous studies, the limit of 4 x 10^4 CFU ml^{-1} in this study is higher than the 10^3 CFU ml^{-1} as reported by both Karsi *et al.* (2006) and Méndez & Guijarro (2013). The luminescence signal is proportional to exposure time i.e. the duration a sample is scanned for (Caliper Life Sciences). In this study fish were scanned for 30 s as oppose to 1 min in the previous studies (Karsi *et al.* 2006; Méndez & Guijarro 2013). We have chosen a shorter exposure time taking into the consideration: 1) uncertainty of the anaesthetic effectiveness over longer durations than 30 s, 2) minimizing chance of obtaining false positives and 3) the overall well-being of the fish.

In this study, fins were suggested as one of the key colonization sites of *A. salmonicida*. This results is in agreement with the findings by Hiney, Kilmartin & Smith (1994), who used ELISA to detect *A. salmonicida* in pre-smolt Atlantic salmon with stress-inducible furunculosis infections. Fins were also found as major colonization sites in other fish pathogens (Martinez, Casado & Enriquez 2004; Harmache, LeBerre, Droineau, Giovannini & Brémont 2006; Menanteau-Ledouble *et al.* 2011). One reason why fins and especially the dorsal fin seem to be an important attachment site could be related to bite wounds (Jobling, Jørgensen & Christiansen 1993). Consistently Svendsen & Bøgwald (1997) found *A. salmonicida* infected salmon with artificial wounds exhibiting higher mortality than infected salmon with no wounds. In contrast, fish used in our study did not have any injuries around the fins during the experiment.

No luminescence emission from the skin was observed in this study and the role of skin as possible colonization site of *A. salmonicida* found in previous studies by Svendsen & Bøgwald (1997), Ferguson *et al.* (1998), Cipriano, Ford, Teska & Hale (1992) and Cipriano, Ford, Schachte & Petrie

(1994) could not be confirmed. Still, given the relatively high threshold limit of luminescence detection, an attachment of a low number of bacteria to the skin cannot be ruled out. An alternative explanation is that teleost (bony) fish feature variation in their immune system (Svendsen, Dalmo & Bøgwald 1999), including varying mucosal activity against pathogens (Dickerson 2009). Rainbow trout could thus have a better mucosal protection against pathogens compared to many other farmed fish species. This hypothesis is supported by other studies where rainbow trout showed the highest degree of resistance against furunculosis compared to other farmed fish species (Cipriano & Heartwell 1986).

A strong BLI signal was also seen around the gills, indicating that this might be an important colonization site as well. This finding is consistent with both Tatner, Johnson & Horne (1984) who investigated *A. salmonicida* infection in rainbow trout and with Svendsen *et al.* (1999) who studied *A. salmonicida* infection in Atlantic salmon. The gills also seem to be an important colonization site for other bacterial fish pathogens like *Yersinia ruckeri*, demonstrated by Ohtani, Villumsen, Strøm & Raida (2014) who observed infection of the gill epithelial cells as early as one minute post infection.

Our study also indicates the oral and nasal cavity and the eyes might be colonization sites for *A. salmonicida*. These three sites are all 'open', i.e. lacking the primary barrier of the skin as a defense against pathogens (Roberts & Ellis 2012). The mouth has also been proposed as a possible entry route for *A. salmonicida* by Svendsen & Bøgwald (1997). Moreover all three sites were seen to be probable colonization sites for *Novirhabdovirus* in a bioluminescence experiment conducted on juvenile trout (Harmache *et al.* 2006). Finally, Karsi *et al.* (2006) reported that bioluminescent *E. ictaluri* became visible around the eye and mouth area during early disease progression.

349

350 At the 4- and 6-hour time points, luminescence was detected in only three of the infected fish,
351 which could, at least in part, be due to the relatively low sensitivity of the method. In two fish, the
352 gills that were found positive at the 2-hour time point were still positive. A luminescence emission
353 signal was observed at the 6-hour time point at the location of the digestive system in one fish,
354 which had not been detected at the 2- and 4-hour time point. Due to the limitation of only being able
355 to acquire two-dimensional images by this method, it was not possible to ascertain whether the
356 luminescence signal came from the inside or the outside of the fish. Though it is likely that the
357 signal came from inside the fish, since the fish had been transferred to clean water after the 2-hour
358 infection immersion time and it would be expected that the bacteria can progress into the fish after a
359 few hours as seen in the study with *Y. ruckeri* (Méndez & Guijarro 2013). In support of this, the
360 luminescence signal intensified at the same location from the 6-hour time point to the 24-hour time
361 point and when the abdominal area was opened revealing the internal organs, a luminescence signal
362 was observed in the intestine (Table 3).

363

364 At the final 24-hour time point, bacteria were seen by imaging in the digestive system and in two
365 fish, also in the spleen. Bacteria were re-isolated from spleen and kidney in all fourteen fish. The
366 reason why bacteria in the kidney and spleen were not detected by imaging could be that the
367 bacteria had lost the pAK*gfplux*1 plasmid, as supported by the lack of fluorescence emission by the
368 re-isolated colonies on blood agar plates, or the bacterial amount was below the threshold detection
369 limit. Dissemination of *A. salmonicida* in the two organs is in agreement with Svendsen *et al.*
370 (1999) who found *A. salmonicida* in blood already after 2 hours post infection and thereafter in
371 kidney and a strong correlation between bacterial amounts in blood and gill tissue samples
372 (Svendsen *et al.* 1999). It is known that bacteria spread from gills to the blood (Dickerson 2009),

which is then filtered by the kidney and spleen (Tatner *et al.* 1984; Hadidi, Glenney, Welch, Silverstein & Wiens 2008).

The imaging results at the 24-hour time point revealed consistently signal form organs after dissection, indicating dissemination of *A. salmonicida* in fish through the digestive system and that this can happen after 24 hours. This scenario is similar to the gut dissemination pattern seen in the study with *Y. ruckeri* (Méndez & Guijarro 2013). Radiolabelled *A. salmonicida* have also previously been found in tissue of the gut (Svendsen *et al.* 1999). In four fish we did not find any luminescent bacteria by 24 hours. Though, at the 2-hour time-point bacteria were seen in three of these fish, indicating that the initial bacteria that entered the digestive system could have passed through the fish in less than 24 hours. This is in agreement with research on digestive responses to feed pellets in rainbow trout, where gastric evacuation was seen after only 4 - 6 hours (Windell, Norris, Kitchell & Norris 1969). Further investigations are needed to shed more light on the role of digestive system in dissemination of *A. salmonicida.*

In summary, our results indicate that the dorsal and pectoral fin and gills are important colonization sites for *A. salmonicida* in rainbow trout. Novel information regarding *A. salmonicida* tissue dissemination pattern was also revealed, including the possible significant role of the digestive system. The bioluminescence-based *A. salmonicida gfplux* model used in the present study provides a valuable tool for *in vivo* real-time imaging of *A. salmonicida* and studying host-pathogen interaction.

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Tables

Table 1. Bacterial CFU ml^{-1} comparison of wild type *A. salmonicida* WT and *A. salmonicida gfplux* after 48 h growth at 20°C, including p-values of a Student's t test, for three experimental repeats.

Experiment	Strain	log_{10} CFU ml^{-1} ± SD	p-value
1	*A. salmonicida* WT	8.85 ± 0.08	0.972
	A. salmonicida gfplux	8.85 ± 0.10	
2	*A. salmonicida WT*	8.51 ± 0.12	0.393
	A. salmonicida gfplux	8.45 ± 0.06	
3	*A. salmonicida WT*	8.69 ± 0.06	0.105
	A. salmonicida gfplux	8.80 ± 0.10	

Table 2. Percent *A. salmonicida gfplux* colonies emitting fluorescence on blood agar plates and Luria Bertani (LB) plates with 100µg ml^{-1} ampicillin. Colonies were re-isolated on agar plates from the kidney, spleen, and brain in fish during the ID50 experiment where fish were injected with different dilutions of *A. salmonicida gfplux* culture.

Blood agar plates		LB plates with 100µg ml^{-1} ampicillin	
Colonies emitting fluorescence¤	**Period (day)**	**Colonies emitting fluorescence¤**	**Period (day)**
30-60 %	3-4	100 %	3-4
10-30 %	5-8	100 % *	5-8
10 > %	9-10	NA #	9-10
0 %	11-14	NA #	11-14

¤ Average percent of fluorescence emitting colonies on a plate, isolated from each organ i.e. kidney, spleen and the brain
***** There were very few colonies on the LB plates compared to the number of colonies on the respective blood agar plates
# There was no growth on the plates

Table 3. Overview of results from the experimental infection. A total of 14 fish infected with *A. salmonicida gfplux* were scanned for 30 s in an IVIS spectrum imaging workstation for detection of luminescence emission at four time points: 2 h, 4 h, 6 h, and 24 h post infection in five independent experiments. Fish from the same experiment are grouped together and given an identification number. All areas of the fish where luminescence was observed at least once are displayed on the left. Observed luminescence signal is presented with an 'X'. No luminescence signal was observed from fish 1.1 and 2.3.

Time point	Area	Fish #													
		1.1	2.1	1.2	2.2	1.3	2.3	1.4a	1.4b	2.4a	2.4b	1.5a	1.5b	2.5a	2.5b
2 h	Caudal fin		X												
	Anal fin											X			
	Dorsal fin		X		X			X				X	X		X
	Pectoral fin		X	X	X								X		
	Anal opening							X							
	Gills		X		X			X	X						
	Nasal cavity		X										X		
	Oral cavity		X										X		
	Eyes		X												
4 h	Gills				X				X						
6 h	Inside of fish					X									
24 h uncut	Anal opening							X							
	Inside of fish					X							X		X
24 h organs	Stomach					X									
	Upper Intestine				X	X				X			X	X	
	Middle Intestine			X	X	X				X	X	X	X	X	X
	Lower Intestine			X		X		X		X			X		X
	Spleen			X*	X*										

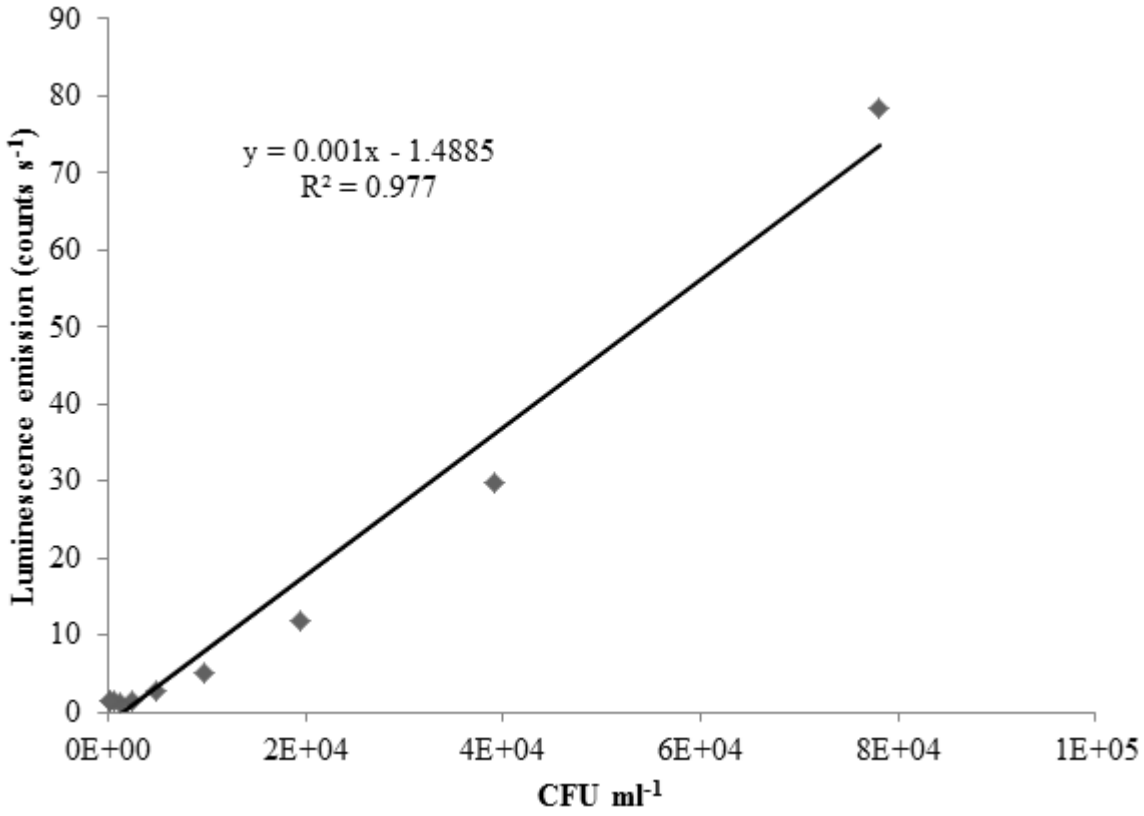
* In a few fish, organ structure at the 24 h time point was deteriorated, making it difficult to distinguish which organ the luminescence signal was coming from, however, it was believed that in two fish the luminescence signal could be emitted from the spleen.

Figure legends

Figure 1. Correlation between measured relative intensity of luminescence emission (counts s^{-1}) and CFU ml^{-1} for *A. salmonicida gfplux* serial dilutions ranging from 8 x 10^4 - 2 x 10^2 CFU ml^{-1} after being scanned for 30 s in an IVIS spectrum imaging workstation. Correlation between CFU ml^{-1} and relative intensity of luminescence emission for *A. salmonicida gfplux* was determined to be linear (R^2 = 0.977) over the range of 8 x 10^4 - 2 x 10^2 CFU ml^{-1}. The correlation coefficient, slope and intercept of the linear regression curve are shown.

Figure 2. Three bioluminescence imaging illustrations from the 2-hour time point of the experimental infection of rainbow trout with *A. salmonicida gfplux*. Illustrations show *A. salmonicida gfplux* colonization of the dorsal and pectoral fin and gills of the following three fish from Table 3: 1.4b, 2.2 and 2.5a.

Figure 3. Six bioluminescence imaging illustrations from the 24-hour time point of the experimental infection of rainbow trout with *A. salmonicida gfplux*. After 24 hours fish were euthanized and visualized in the IVIS as whole fish and were then cut open. In uncut fish 1.3, luminescence signal was observed in the body organ area and when cut open, the signal was observed in the stomach and upper, middle and lower intestine. In uncut fish 1.5b, luminescence signal was observed in the body organ area and when cut open, the signal was observed in the upper, middle and lower intestine. In uncut fish 1.4a, luminescence signal was observed around the anal opening and when cut open, the signal was observed around the anal opening and in the lower intestine.



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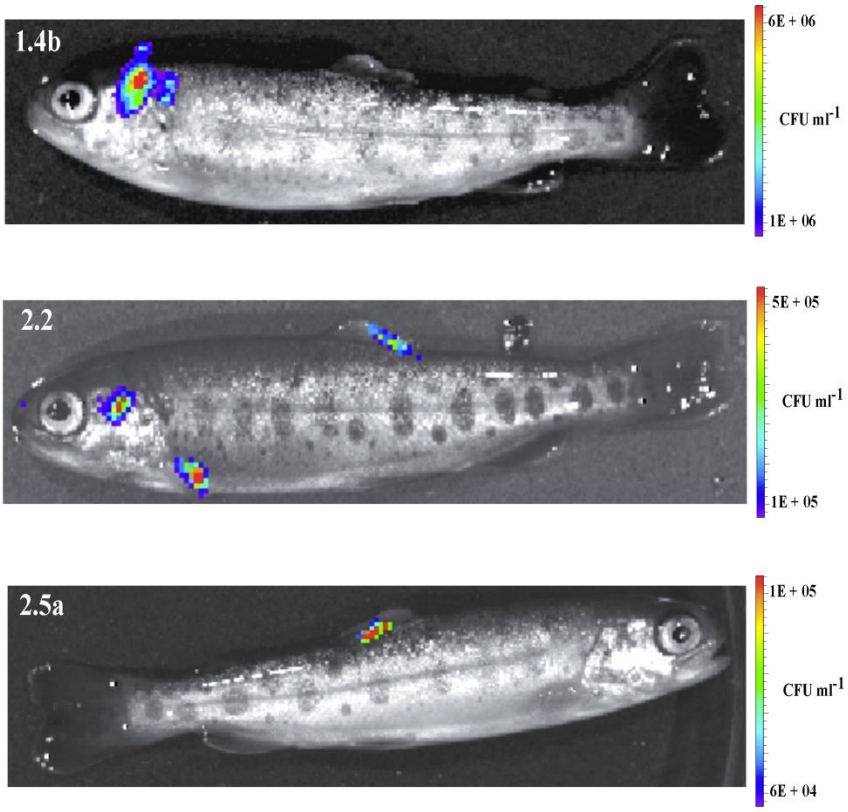

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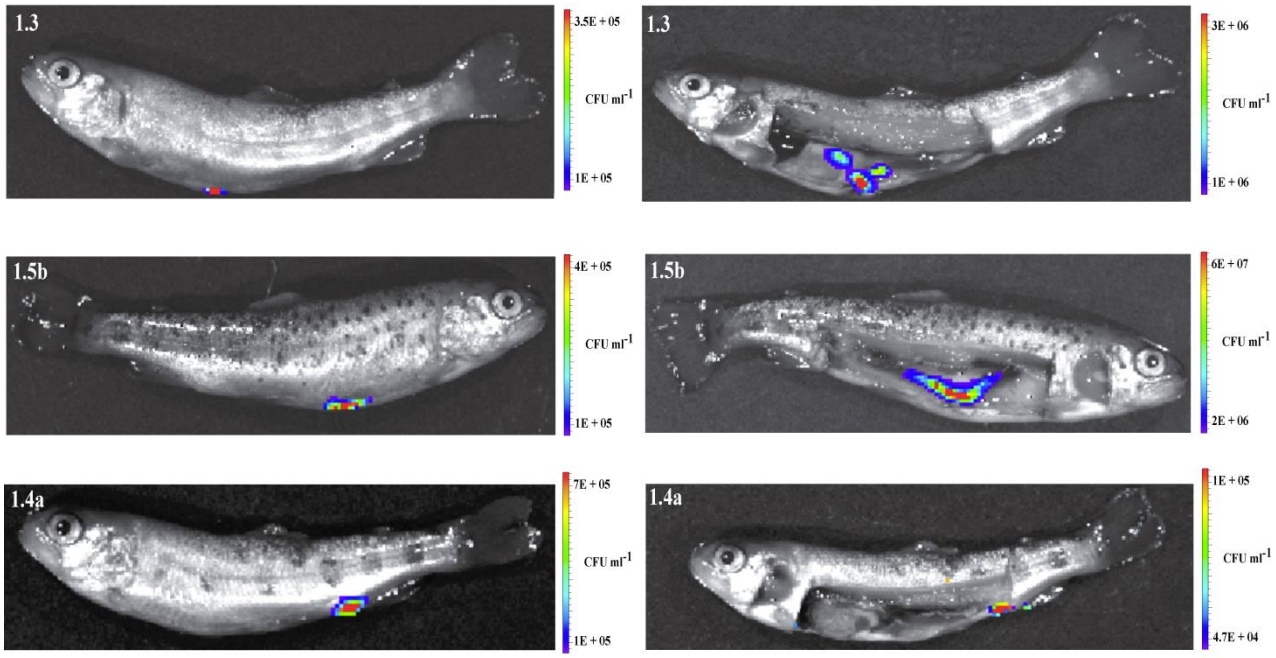

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Manuscript II

Detection and quantification of *Aeromonas salmonicida* in fish tissue by real-time PCR

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Detection and quantification of *Aeromonas salmonicida* in fish tissue by real-time PCR

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Abstract

Furunculosis, a septicaemic infection caused by the bacterium *Aeromonas salmonicida* subsp. *salmonicida*, currently causes problems in Danish seawater rainbow trout production. Detection has mainly been achieved by bacterial culture, but more rapid and sensitive methods are needed. A previously developed real-time PCR assay targeting the plasmid encoded *aopP* gene of *A. salmonicida* was, in parallel with culturing, used for the examination of five organs of 40 fish from Danish freshwater and seawater farms. Real-time PCR showed overall a higher frequency of positives than culturing (65% of positive fish by real-time PCR compared to 30% by a culture approach). Also, no real-time PCR-negative samples were found positive by culturing. *A. salmonicida* was detected by real-time PCR, though not by culturing, in freshwater fish showing no signs of furunculosis, indicating possible presence of carrier fish. In seawater fish examined after an outbreak and antibiotics treatment, real-time PCR showed the presence of the bacterium in all examined organs (1–482 genomic units mg^{-1}). With a limit of detection of 40 target copies (1–2 genomic units) per reaction, a high reproducibility and an excellent efficiency, the present real-time PCR assay provides a sensitive tool for the detection of *A. salmonicida*.

Keywords: Aeromonas salmonicida, *aopP*, furunculosis, pAsal1, rainbow trout, real-time PCR.

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Introduction

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of furunculosis, a septicaemic infection that over the years has caused worldwide losses in aquaculture (O'Brien, Mooney, Ryan, Powell, Hiney, Kilmartin & Smith 1994; Beaz-Hidalgo & Figueras 2012). In Denmark, the infection was first described in freshwater during the 1950s by Rasmussen (1964). Today, problems with furunculosis in Denmark occur mainly in sea-reared rainbow trout (*Oncorhynchus mykiss*) production under stressful conditions and high temperatures (Dalsgaard & Madsen 2000; Pedersen *et al.* 2008). The presence of *A. salmonicida* in fish does not necessarily lead to the development of furunculosis, although some fish may be carriers that transfer *A. salmonicida* from freshwater to the sea (Jarp *et al.* 1993; Dalsgaard & Madsen 2000).

Detection of *A. salmonicida* has usually been performed by the use of bacterial cultivation (Dalsgaard & Madsen 2000; Austin & Austin 2007). However, the detection of the bacterium from supposed carrier fish with use of this method has not been successful so far (Dalsgaard & Madsen 2000). Although employing pre-enrichment steps or subjecting the fish to stress improves the detection of *A. salmonicida* by culturing (Cipriano *et al.* 1997), more sensitive methods are needed for the evaluation of the presence of *A. salmonicida* in carriers.

Real-time PCR has been used in several studies to detect *A. salmonicida* in fish tissue (Balcazar *et al.* 2007; Goodwin & Merry 2009; Keeling

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et al. 2012; Gulla *et al.* 2015). In the study by Balcazar *et al.* (2007), an assay was developed with 100% specificity and a sensitivity of 16 CFU per reaction. The primers target an *A. salmonicida* DNA probe sequence from a 6.4-kb *A. salmonicida* plasmid named pAsal1 by Fehr *et al.* (2006), which has been the most frequently used target for species-specific *A. salmonicida* PCR and real-time PCR assays to date (Hiney *et al.* 1992; Morgan, Rhodes & Pickup 1993; O'Brien *et al.* 1994; Mooney *et al.* 1995; Byers, Gudkovs & Crane 2002; Balcazar *et al.* 2007; Goodwin & Merry 2009).

The objective was to investigate whether the real-time PCR assay developed by Balcazar *et al.* (2007), employed on multiple organs, would provide a more sensitive tool than bacterial culturing for determining *A. salmonicida* prevalence in rainbow trout from freshwater and seawater farms showing no signs of disease and from one seawater farm after a furunculosis outbreak. Samples included spleen, kidney, intestine, gill and brain tissues from 40 fish, sampled at five freshwater and seawater farms over the course of 2 years.

Materials and methods

Bacterial strains

To examine distribution of the pAsal1 plasmid and the target gene *aopP* in natural populations of *A. salmonicida* in Denmark, 20 Danish strains, isolated from outbreaks in fresh and seawater farms at different geographical locations in the period 1984–2014, were included in the study (data not shown). The strains were grown in Veal Infusion Broth (VIB) (Difco) for 48 h at 20°C. DNA was extracted with Qiagen QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol and immediately stored at −20°C until further use.

Development of standard

An *A. salmonicida* DNA standard used for production of the standard curve was made from cloned PCR fragment of the target gene (*aopP*). PCR primers 5′ TAGCTGGTTCCATAAGAA GC 3′ and 5′ TCCAAGAGGCAACTAAAGAAG 3′ flanking the target sequence of the real-time PCR LUX primers developed by Balcazar *et al.* (2007), ensuring that both the real-time PCR LUX target and primer sequences would be included in the amplified fragment, were generated from *A. salmonicida* pAsal1 plasmid sequence (GenBank accession no. AJ508382) and used to amplify a 340-bp fragment of the *aopP* gene from extracted DNA of *A. salmonicida* type strain ATCC 33658. The PCR product was then purified using the High Pure PCR Product Purification kit (Roche Applied Science) and cloned using a pGEM®-T and pGEM®-T Easy Vector Systems cloning kit (Promega) according to the manufacturer's instructions. Subsequently, the vector plasmid was purified with Qiagen Plasmid Midi Kit (Qiagen) and DNA concentration was determined using a Qubit 2.0 fluorometer and Quant-iT dsDNA BR kit (Invitrogen). Insertion of the 340-bp *aopP* gene fragment into the plasmid vector was verified by amplification with pUC/M13 universal primers (Promega) and sequencing of the amplicon using an ABI 3130 Genetic Analyser and Big Dye Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's instruction.

Real-time PCR

Real-time PCR was carried out in a total of 25 μL volume, containing 12.5 μL of 2X JumpStart Taq ReadyMix for quantitative PCR (Sigma-Aldrich), 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 μM solution of each LUX PCR primer (Balcazar *et al.* 2007), 8 μL of nuclease-free water and 2 μL of DNA template. The thermal cycling conditions included an initial step at 95°C for 2 min, followed by 40 cycles consisting of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. Amplification and data analysis were performed using a Rotor-Gene Q system (Qiagen) and software version 2.0.2. All real-time PCR runs included template-free negative controls and positive *A. salmonicida* DNA standards consisting of selected dilutions. Samples were considered negative if no amplification signal was produced. Samples were considered positive if an amplification signal was produced in at least one of the triplicates and verified with melting point analysis.

Sensitivity, reproducibility and dynamic range of real-time PCR

In order to create a standard curve, a solution of plasmid vector containing 14.3 ng μL^{-1} of DNA

was used to prepare nine 10-fold serial dilutions in nuclease-free water down to 1.43 fg μL^{-1}, corresponding to a dilution range of approximately 4×10^9 to four plasmid copies per μL. Two microlitres of each dilution was used as template in three independent real-time PCRs with three replicates of each dilution.

To determine limit of detection (LOD) and quantification limit (QL), 2 μL of plasmid vector containing 80, 40, 20 and 10 copies of plasmid, respectively, was amplified. The obtained data were then used to graph the linear regression for the standard curve plot with the LOD point using RStudio (2015). The LOD was defined as the lowest DNA concentration (target gene copies per reaction) at which 95% of the positive samples were detected. The QL was defined as the lowest DNA concentration that remained within the linear region of the standard curve concentrations. Given an average of 20–50 pAsal1 plasmid copies per cell (Fehr *et al.* 2006; Attéré *et al.* 2015), quantitative real-time PCR data were transformed to express the results as genomic copies (genomic units, GU).

Reproducibility of the real-time PCR was assessed by calculating the interassay variance coefficient (CV%) in MS Excel (Microsoft). Calculation was based on the mean log of DNA copies per reaction, generated in three independent real-time PCR runs on the eight 10-fold serial dilution of plasmid vector DNA.

Analysis of tissue inhibition in real-time PCR

An effect of possible co-purified inhibitors from the host tissues on sensitivity of the PCR assay was examined by amplification of gill, intestine, brain, kidney and spleen tissues seeded with *A. salmonicida* ATCC 33658.

Rainbow trout fry originating from eggs from Fousing Trout Farm that were disinfected and hatched at AquaBaltic and brought to our institute for rearing were used for collection of tissue. Six fish were killed in 5 g L^{-1} of Tricaine Methanesulfonate MS-222 (Sigma-Aldrich) in accordance with regulations set forward by the Danish Ministry of Justice and Animal Protection committees and under the Danish Animal Experiments Inspectorate permit number 2012-15-2924-00629. Spleen, gills, intestine, brain and kidney were collected under aseptic conditions and immediately stored at −20°C until further use.

Seeding of tissues was performed as follows: *A. salmonicida* ATCC 33658 was grown in VIB at 20°C for 48 h. Serial 10-fold dilutions of bacterial cells containing 5×10^8 to 5×10^2 CFU mL^{-1} were prepared in 0.9% sterile saline solutions and counted on blood agar plates. In addition, two extra dilutions containing 2.5×10^2 and 1.25×10^2 CFU mL^{-1} were prepared, making up a total of nine different concentrations of bacterial cells. Subsequently, 1 mL of each bacterial cell concentration was mixed with 40 mg of gill, intestine, brain or kidney tissue or 15 mg of spleen tissue. All samples were then homogenized at 30 Hz for 20 s in a TissueLyser II (Qiagen), and DNA was prepared for amplification using the InstaGene Matrix (Bio-Rad) according to the manufacturer's instruction. Dilutions of bacterial cells without addition of any tissue and homogenized tissue with 0.9% saline solution were also included as positive and negative controls, respectively. The whole seeding experiment was repeated three times.

The real-time PCR data were analysed using Rotor-Gene Q Series Software (Qiagen) and used to graph the linear regression for organ tissue dilution series using RStudio (2015). The LOD and QL for each organ tissue were defined as for pure bacterial culture. For each seeding experiment, obtained log copies of seeded specimens and bacterial culture not mixed with any tissue were compared by ANOVA using RStudio (2015).

Detection of *A. salmonicida* in fish

Rainbow trout (n = 40) with and without signs of disease were collected for testing with bacterial culturing and real-time PCR from November 2013 through November 2015 at three freshwater and two seawater farms in Denmark. Twenty of the 40 fish were collected from one of the seawater farms (no. 5) 5 days after an antibiotic treatment of furunculosis and again at slaughtering 3 months after the first sampling. Between the two samplings, antibiotic treatment of fish was repeated. Sampling from farm no. 1, 2, 3 and 5 consisted of the same batch of fish that had been followed throughout the 2 years.

The spleen, gills, intestine, brain and kidney were removed from all fish and placed in individual Eppendorf tubes. Each sample was inoculated onto a blood agar plate (Columbia agar base [Oxoid] with 5% calf blood) by dipping an

inoculation loop into the tissue and then plating the content onto the blood agar, which was incubated at 20°C for 48–72 h (Dalsgaard & Madsen 2000). The remaining organ tissues were immediately stored at −20°C until DNA extraction for real-time PCR.

For DNA extraction, 20–40 mg specimens of the gill, intestine, brain and kidney and 10–15 mg from the spleen were homogenized with 1 mL of nuclease-free water at 30 Hz for 20 s in a TissueLyser II (Qiagen). Extraction was performed using the InstaGene Matrix (Bio-Rad) kit according to the manufacturer's protocol. Extracted DNA was immediately stored at −20°C until further use. All samples and positive and negative control samples were run in triplicates with real-time PCR. Rotor-Gene Q Series Software, version 2.0.2 (Qiagen), was used for analysis of the data.

Results

Specificity, sensitivity and reproducibility of real-time PCR

All examined Danish *A. salmonicida* strains ($n = 20$) isolated from the outbreaks of furunculosis over a 30-year period, tested positive with real-time PCR (results not shown).

Amplification of standard DNA templates showed a broad linear dynamic range, spanning from 8×10^9 to 8×10^1 target copies, with a slope of −3.28 ($R^2 = 0.994$) and an efficiency of 102% (Fig. 1). The LOD was found to be 40 target copies per reaction with a quantification cycle (C_q) of 38.32 ± 0.73, which is equivalent to 1–2 *A. salmonicida* GU per reaction. The QL was found to be 80 target copies per reaction (C_q 37.66 ± 0.56) equivalent to 2–4 *A. salmonicida* GU per reaction. The interassay CV for three independent real-time PCR runs was 0.94%.

Analysis of tissue inhibition in real-time PCR

No evidence of inhibition was found for 40 mg of organs (15 mg for spleen) over the broad range of *A. salmonicida* ATCC 33658 concentrations. Dilution series for all five organs showed a linear trend from 5×10^8 to 2.5×10^2 CFU mL^{-1} (Fig. 2), and assay results corresponded well to mean CFU mL^{-1} values (results not shown). No significant difference was observed when comparing log copies per reaction obtained by real-time

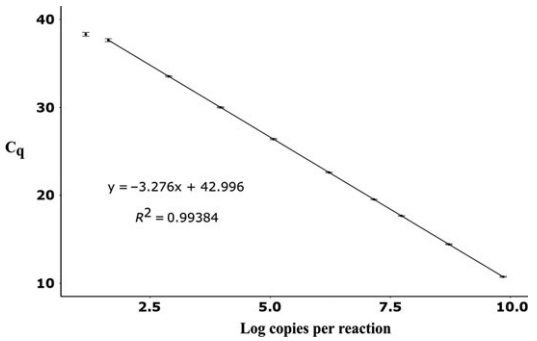

Figure 1 Standard curve created by plotting the mean quantification cycle (C_q) values as a function of log target copies per reaction of the plasmid vector dilutions. Ten-fold serial dilutions ranging from 14.3 ng μL^{-1} to 1.43 fg μL^{-1} of the plasmid vector were analysed in three independent runs performed on the Rotor-Gene Q with three replicates per dilution concentration. The correlation coefficient, slope and intercept of the regression curve are shown. Error bars represent standard deviations (SD) of *Aeromonas salmonicida* standard log copies per reaction. The limit of detection (LOD) with a mean C_q of 38.32 and 1.61 ± 0.22 SD log target copies per reaction is plotted, but lies outside of the linear region of the standard curve and is therefore not connected to the linear regression line.

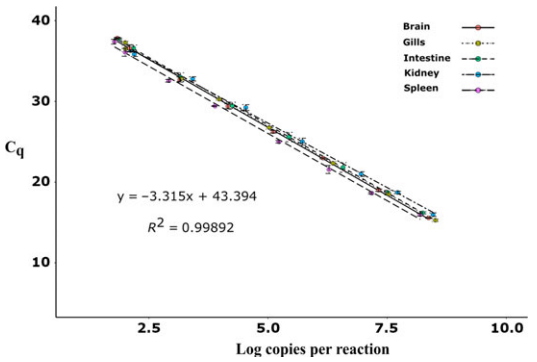

Figure 2 Regression curves for the *Aeromonas salmonicida* real-time PCR assay for five seeded organ tissues, created by plotting the mean quantification cycle (C_q) values as a function of log target copies per reaction of the *A. salmonicida* ATCC concentrations added to the respective tissues. Serial dilutions ranging from 5×10^8 to 2.5×10^2 CFU mL^{-1} of *A. salmonicida* ATCC 33658 were added to five different tissues: intestine, kidney, brain, spleen and the gills. DNA was extracted and analysed in three independent runs performed on the Rotor-Gene Q with three replicates per dilution concentration. The combined mean of the correlation coefficients, slopes and intercepts of the five regression curves is shown. Error bars represent standard deviations (SD) of *A. salmonicida* ATCC 33658 log target copies per reaction.

PCR from dilutions of *A. salmonicida* ATCC 33658 broth culture vs. dilutions of ATCC 33658 culture added to tissue using ANOVA ($P > 0.05$). All negative control tissue samples were found negative by the real-time PCR.

Detection of *A. salmonicida* in fish

In fish from the three freshwater farms (no. 1–3), no *A. salmonicida* were detected by the culture method (Table 1). However, *A. salmonicida* was detected in four of the 18 examined fish by real-time PCR (Table 1). In two cases, *A. salmonicida* was detected in both the brain and spleen and in the two other cases the bacterium was detected in either the brain or spleen. Plasmid copy number was in the range of 103–402 mg^{-1} which equals from 2 up to 20 GU mg^{-1}.

At seawater farm no. 4, one of the two examined fish was positive for *A. salmonicida* in one organ (the brain) by culturing (Table 1). The real-time PCR detected *A. salmonicida* in both fish and several organs: the spleen and intestine of one fish and the spleen, intestine and brain of the other fish with a plasmid copy number in the range of 137–736 mg^{-1}, which equals from 2 up to 37 GU mg^{-1}.

At fish farm no. 5 (Table 2) sampled 5 days after antibiotics treatment, *A. salmonicida* was detected in nine of 16 fish by culturing, but was detected in all 16 fish and in 73 of 80 organ samples by real-time PCR. Plasmid copy numbers were in the range of 21–9638 mg^{-1}, which equals from 1 up to 482 GU mg^{-1}. At the second sampling, 3 months after the first sampling, *A. salmonicida* was detected in two of the four examined fish by culturing. All fish and organ samples were, however, found positive by real-time PCR with a plasmid copy number in the range of 77–2364 mg^{-1}, which equals from 1 up to 118 GU mg^{-1}.

Overall, culturing detected *A. salmonicida* in 12 of the 40 examined fish, while real-time PCR detected *A. salmonicida* in 26 fish.

Discussion

Specificity for the assay target gene *aopP* located on the plasmid pAsal1 was found to be 100% in the studies by Balcazar *et al.* (2007), who examined 16 *A. salmonicida* and 26 non- *A. salmonicida* bacterial strains, and by Goodwin & Merry (2009) that tested six *A. salmonicida* strains. In the present study, all 20 Danish *A. salmonicida* strains that were examined with the assay were amplified, thereby suggesting a 100% prevalence of the *aopP* gene. Earlier studies have shown that plasmid pAsal1 is absent from some *A. salmonicida* strains (Morgan *et al.* 1993; Sørum, Kvello & Hastein 1993; O′Brien *et al.* 1994; Mooney *et al.* 1995; Byers *et al.* 2002), including 25% of 57 examined Danish strains (Nielsen, Olsen & Larsen 1993). Some authors explain the missing plasmid by stressful culturing conditions above 22–25°C, which was shown to activate an pAsal1 insertion sequence element IS*AS11* that leads to the loss of the plasmid (Daher *et al.* 2011; Tanaka *et al.* 2012; Attéré *et al.* 2015). Others, such as Balcazar *et al.* (2007), have argued that the presence of pAsal1 could be associated with virulence of the bacterium and *A. salmonicida* lacking this plasmid might thus be less virulent. However, neither stressful culturing nor lack of virulence is necessarily associated with the absence of pAsal1 (Boyd *et al.* 2003; Fehr *et al.* 2006; Attéré *et al.* 2015), and one must keep this in mind when using *aopP* as the target gene.

Plasmid pAsal1 is present in 20–50 copies per bacterial cell (Fehr *et al.* 2006; Attéré *et al.* 2015), and the high abundance of the target facilitates detection by real-time PCR. We were able to reliably detect 1–2 *A. salmonicida* GU per reaction. Four positive samples had lower values than 1 GU per reaction (Table 2), but were still considered positive based on correct melting point of the obtained PCR product, despite being slightly below the 95% reliability. Although the plasmid copy variation per cell presents an obstacle for precise quantification of GU, it has little importance for practical application of the assay. The drawback of pAsal1 is that the plasmid is not universally present in all isolates of *A. salmonicida*. However, our results indicate that the pAsal1 plasmid is present in isolates causing new outbreaks in Danish fish farms and the frequent application of this target for *A. salmonicida* detection around the world enables comparison of results with the bulk of the published literature (Hiney *et al.* 1992; Morgan *et al.* 1993; O′Brien *et al.* 1994; Mooney *et al.* 1995; Byers *et al.* 2002; Balcazar *et al.* 2007; Goodwin & Merry 2009).

Compared to real-time PCR by Balcazar *et al.* (2007), sensitivity obtained in the present study was higher. Balcazar *et al.* (2007) achieved a LOD/QL of 16 *A. salmonicida* CFU per reaction

Table 1 Detection and quantification of *Aeromonas salmonicida* from 12 fish sampled from three freshwater (no. 1–3) and one seawater fish farm (no. 4) in Denmark during 2013–2015

					Real-time PCR		
Fish farm	Sampling date (yyyy.mm.dd)	Fish ID	Tissue	Bacteriology	Target copies mg^{-1}	Log target copies $mg^{-1} \pm SD$	$C_q \pm SD$
No. 1	2013.11.04	3	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		4	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		11	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		15	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		26	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		35	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
No. 2	2014.02.18	48	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		76	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
	2014.05.14	83	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		95	Gills	–	–	–	–
			Spleen	–	402	2.60 ± 0.08	36.87 ± 0.26
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	107	2.03 ± 0.22[b]	38.06 ± 0.72[b]
		105	Gills	–	–	–	–
			Spleen	–	198	2.30 ± 0.13[ab]	38.06 ± 0.41[ab]
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	159	2.20 ± 0.17	37.44 ± 0.57[b]
		113	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–

Table 1 *Continued*

Fish farm	Sampling date (yyyy.mm.dd)	Fish ID	Tissue	Bacteriology	Real-time PCR		
					Target copies mg^{-1}	Log target copies $mg^{-1} \pm SD$	$C_q \pm SD$
			Brain	–	–	–	–
No. 3	2014.11.03	122	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	103	2.02 ± 0.27[b]	37.95 ± 0.89[b]
		148	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
	2015.03.24	170	Gills	–	–	–	–
			Spleen	–	233	2.37 ± 0.33[b]	38.26 ± 1.08[b]
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
			Gills	–	–	–	–
			Spleen	–	–	–	–
		184	Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		185	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
		198	Gills	–	–	–	–
			Spleen	–	–	–	–
			Intestine	–	–	–	–
			Kidney	–	–	–	–
			Brain	–	–	–	–
No. 4	2015.11.04	1	Gills	–	–	–	–
			Spleen	–	443	2.65 ± 0.26	37.11 ± 0.86
			Intestine	–	137	2.14 ± 0.21	37.54 ± 0.69
			Kidney	–	–	–	–
			Brain	–	–	–	–
		3	Gills	–	–	–	–
			Spleen	–	763	2.88 ± 0.07	36.34 ± 0.22
			Intestine	–	258	2.41 ± 0.22	37.01 ± 0.71
			Kidney	–	–	–	–
			Brain	+	231	2.33 ± 0.03	36.61 ± 0.10

–, Examined with negative result.
[a]Only two of three sample replicates produced an amplification signal.
[b]Quantification cycle (C_q) value below quantitation limit (QL). Reported quantification value of copies mg^{-1} could be imprecise.

in pure culture as well as inoculated tissue. In comparison, the QL and LOD of the present assay were 80 and 40 target genes copies per reaction, respectively, equivalent to, respectively, 2–4 and 1–2 *A. salmonicida* GU per reaction. An explanation for the difference in sensitivity between the present study and Balcazar *et al.* (2007), even though the same assay is applied in both studies, could be that another real-time PCR instrument and kit was used in this study and the standard curve was generated from a cloned PCR product, while Balcazar *et al.* (2007) used extracted DNA from pure cultures of *A. salmonicida* NCIMB 1102.

The most frequently examined organ for *A. salmonicida* in previous real-time PCR studies has been the kidney (Balcazar *et al.* 2007; Goodwin & Merry 2009; Keeling *et al.* 2012; Gulla *et al.* 2015). In the present study, four additional organs were included: spleen, intestine, gills and the brain. Interestingly, in all four real-time PCR-positive freshwater farm fish, the brain and/or the spleen were found positive but not the kidney (Table 1). In comparison, no *A. salmonicida* was

Table 2 Detection and quantification of *Aeromonas salmonicida* from 20 fish sampled 11th of August and 11th of November 2015 at seawater farm no. 5. These samples were taken 5 days and 3 months after antibiotic treatment of furunculosis

					Real-time PCR		
Fish farm	Sampling date (yyyy.mm.dd)	Fish ID	Tissue	Bacteriology	Target copies mg^{-1}	Log target copies mg^{-1} ± SD	C_q ± SD
No. 5	2015.08.11	9	Gills	–	171	2.23 ± 0.55	36.32 ± 1.80
			Spleen	–	220	2.34 ± 0.13	37.44 ± 0.43
			Intestine	–	510	2.71 ± 0.20	34.61 ± 0.65
			Kidney	–	473	2.67 ± 0.18	35.17 ± 0.58
			Brain	–	417	2.62 ± 0.25	34.89 ± 0.82
		13	Gills	+	3732	3.57 ± 0.06	37.73 ± 0.21
			Spleen	–	2276	3.36 ± 0.02	33.57 ± 0.06
			Intestine	–	–	–	–
			Kidney	–	1577	3.20 ± 0.27	32.95 ± 0.87
			Brain	–	669	2.83 ± 0.48	33.54 ± 1.58
		15	Gills	–	92	1.96 ± 0.11	37.15 ± 1.00
			Spleen	–	2526	3.40 ± 0.01	33.77 ± 0.77
			Intestine	–	562	2.75 ± 0.16	34.34 ± 0.79
			Kidney	–	283	2.45 ± 0.05	35.26 ± 0.16
			Brain	–	54	1.73[b]	37.28[b]
		17	Gills	+	968	2.68 ± 0.20	35.70 ± 0.66
			Spleen	–	2050	3.31 ± 0.12	33.29 ± 0.38
			Intestine	–	14	1.15[bd]	39.23[bd]
			Kidney	–	825	2.92 ± 0.09	33.74 ± 0.30
			Brain	–	702	2.85[b]	33.68[b]
		22	Gills	–	38	1.51 ± 0.35[ac]	37.99 ± 1.15[ac]
			Spleen	–	1071	3.02 ± 0.13[a]	34.58 ± 0.42[a]
			Intestine	–	198	2.3[b]	35.34[b]
			Kidney	–	–	–	–
			Brain	–	277	2.44 ± 0.19	34.99 ± 0.62
		23	Gills	+	146	2.17 ± 0.44	36.44 ± 1.45
			Spleen	–	766	2.88 ± 0.21	35.42 ± 0.70
			Intestine	–	473	2.67 ± 0.23	34.97 ± 0.76
			Kidney	–	7286	3.86 ± 0.20	32.11 ± 0.64
			Brain	–	432	2.64 ± 0.32	34.83 ± 1.06
		25	Gills	–	–	–	–
			Spleen	–	722	2.86 ± 0.12	36.03 ± 0.39
			Intestine	–	34	1.53 ± 0.21[ad]	39.03 ± 0.67[ad]
			Kidney	–	227	2.36 ± 0.34	37.02 ± 1.11
			Brain	–	49	1.69 ± 0.05[ad]	38.63 ± 0.17[ad]
		26	Gills	–	44	1.63[bd]	38.69[bd]
			Spleen	–	203	2.31 ± 0.17[c]	38 ± 0.57[c]
			Intestine	–	21	1.32[bd]	39.99[bd]
			Kidney	–	122	2.09 ± 0.26[c]	37.89 ± 0.85[c]
			Brain	–	–	–	–
		28	Gills	–	–	–	–
			Spleen	–	933	2.97 ± 0.18	35.51 ± 0.58
			Intestine	–	–	–	–
			Kidney	–	1189	3.08 ± 0.13	34.46 ± 0.41
			Brain	–	1344	3.13 ± 0.12	33.63 ± 0.40
		29	Gills	+	86	1.93 ± 0.26	36.68 ± 0.84
			Spleen	+	882	2.95 ± 0.05	35.06 ± 0.15
			Intestine	–	24	1.37 ± 0.23[ad]	39.07 ± 0.76[ad]
			Kidney	+	943	2.97 ± 0.46	34.12 ± 1.51
			Brain	–	591	2.77 ± 0.21	34.35 ± 0.68
		30	Gills	–	493	2.69 ± 0.26	34.80 ± 0.84
			Spleen	–	5106	3.71 ± 0.35	32.54 ± 1.13
			Intestine	–	134	2.13 ± 0.43	36.58 ± 1.42
			Kidney	–	1436	3.00 ± 0.56[a]	34.21 ± 1.82[a]
			Brain	+	1907	3.28 ± 0.15	32.71 ± 0.48
		31	Gills	+	173	2.24 ± 0.06	36.59 ± 0.21
			Spleen	–	9638	3.98 ± 0.07	31.87 ± 0.23
			Intestine	–	345	2.54 ± 0.20	35.33 ± 0.66
			Kidney	–	957	2.98 ± 0.23	34.73 ± 0.76

Table 2 *Continued*

Fish farm	Sampling date (yyyy.mm.dd)	Fish ID	Tissue	Bacteriology	Real-time PCR: Target copies mg^{-1}	Real-time PCR: Log target copies mg^{-1} ± SD	Real-time PCR: C_q ± SD
			Brain	–	755	2.88 ± 0.17	34.25 ± 0.57
		32	Gills	+	241	2.38 ± 0.15	36.14 ± 0.49
			Spleen	+	3611	3.56 ± 0.18	33.59 ± 0.60
			Intestine	–	1185	3.07 ± 0.15	33.66 ± 0.50
			Kidney	+	821	2.91 ± 0.17	34.62 ± 0.56
			Brain	+	3463	3.54 ± 0.11	32.23 ± 0.36
		37	Gills	–	–	–	–
			Spleen	–	2565	3.41 ± 0.21	34.30 ± 0.70
			Intestine	–	288	2.46 ± 0.20	36.29 ± 0.67
			Kidney	–	110	2.04 ± 0.16[c]	37.58 ± 0.53[c]
			Brain	–	300	2.48 ± 0.32	35.89 ± 1.04
		39	Gills	+	65	1.81 ± 0.62[c]	37.86 ± 2.04[c]
			Spleen	–	7930	3.90 ± 0.07	32.63 ± 0.24
			Intestine	–	631	2.80 ± 0.28	34.76 ± 0.92
			Kidney	–	448	2.65 ± 0.13	35.45 ± 0.41
			Brain	–	299	2.48 ± 0.30	35.69 ± 0.98
		42	Gills	+	288	2.46 ± 0.21	35.81 ± 0.70
			Spleen	+	1336	3.13 ± 0.33	34.82 ± 1.09
			Intestine	–	168	2.23 ± 0.14	36.52 ± 0.47
			Kidney	+	1937	3.29 ± 0.11	33.74 ± 0.35
			Brain	–	338	2.53 ± 0.07	35.51 ± 0.23
	2015.11.11	3	Gills	–	185	2.27 ± 0.24	37.16 ± 0.85
			Spleen	–	1019	3.01 ± 0.16	36.24 ± 0.56
			Intestine	–	299	2.48 ± 0.11	36.39 ± 0.37
			Kidney	–	143	2.16 ± 0.46	37.60 ± 1.61
			Brain	–	216	2.33 ± 0.20	37.13 ± 0.69
		8	Gills	+	222	2.35 ± 0.1	36.94 ± 0.34
			Spleen	–	441	2.64[b]	36.69[b]
			Intestine	–	456	2.66 ± 0.04	35.71 ± 0.13
			Kidney	+	1322	3.12 ± 0.15	34.87 ± 0.52
			Brain	–	341	2.53 ± 0.40	36.06 ± 1.41
		23	Gills	–	183	2.26 ± 0.53	37.49 ± 1.85
			Spleen	–	2111	3.32 ± 0.20	34.48 ± 0.69
			Intestine	–	77	1.89 ± 0.57[ad]	38.45 ± 1.98[ad]
			Kidney	+	162	2.21 ± 0.16[c]	38.06 ± 0.56[c]
			Brain	–	190	2.28 ± 0.34	36.95 ± 1.18
		27	Gills	–	126	2.10 ± 0.21[ac]	37.62 ± 0.75[ac]
			Spleen	–	2364	3.37 ± 0.08	34.22 ± 0.28
			Intestine	–	670	2.83 ± 0.11	35.90 ± 0.38
			Kidney	–	481	2.68 ± 0.15	36.14 ± 0.51
			Brain	–	785	2.90 ± 0.19	34.80 ± 0.66

–, Examined with negative result.
[a]Only two of three sample replicates produced an amplification signal.
[b]Only one of three sample replicates produced an amplification signal.
[c]Quantification cycle (C_q) value below quantitation limit (QL). Reported quantification value of copies mg^{-1} could be imprecise.
[d]Quantification cycle (C_q) value below limit of detection (LOD). Reported quantification value of copies mg^{-1} has to be taken tentatively.

isolated by bacterial culturing in any of the 18 fish from freshwater farms as well as in none of the additional 182 fish sampled from the same three freshwater farms (data not shown). None of the freshwater farm fish showed any signs of furunculosis, indicating that the four fish found positive by real-time PCR could be carriers of viable but non-culturable cells (VBNC) (Morgan *et al.* 1993; Ferguson *et al.* 1995; Nascutiu 2010). Due to the high sensitivity of the present real-time PCR, one could speculate whether the four fish could be false positives by contamination of the surrounding water. However, in that case one would presume the gills would be the primarily infected organs, while in the present study; the gills were negative in all fish showing no signs of disease. In addition, none of the previous studies that have employed the pAsal1 target have detected other bacterial DNA than that of *A. salmonicida* (Hiney *et al.* 1992; Morgan *et al.* 1993; O'Brien *et al.*

1994; Mooney *et al.* 1995; Byers *et al.* 2002; Balcazar *et al.* 2007; Goodwin & Merry 2009).

It was reported by Gustafson, Thomas & Trust (1992) that in farmed brown trout (*Salmo trutta*) up to 80% of the fish are thought to be carriers of *A. salmonicida* and VBNC *A. salmonicida* have also been revived (Austin, Austin & Colwell 1984). The presence of VBNC *A. salmonicida* would also explain why the bacterium has not been found by culturing in the Danish rainbow trout freshwater farms, although furunculosis outbreaks occur during elevated temperatures after fish are transferred to the sea (Dalsgaard & Madsen 2000; Pedersen *et al.* 2008). Our results support this hypothesis, as the fish sampled at three freshwater farms represented the same batch of fish. Initially, fish were hatched at freshwater farm no. 1, then transferred to freshwater farm no. 2 where *A. salmonicida* became detectable by real-time PCR, then further transferred to freshwater farm no. 3 still being *A. salmonicida* positive by real-time PCR, and finally transferred to seawater farm no. 5 at 2 years age, where furunculosis outbreak occurred later that year. Further studies are underway to shed a light on possible transmission of the disease.

In fish reared in sea water, *A. salmonicida* was detected by culturing in 12 of 22 fish and 20 of 110 organs, while the bacterium was detected in all 22 fish and 98 organs by real-time PCR (Table 1 and 2). This substantial difference in detection between the culturing and real-time PCR approach might be explained, at least in part, by the fact that 20 fish accounting for 93 of the positive organs were obtained from farm no. 5. At this farm, there had been a furunculosis outbreak, but unfortunately, it was not possible to do the sampling until 5 days after the fish had been treated with antibiotics. The treatment most likely either reduced or killed *A. salmonicida* cells in the fish, thereby reducing or eliminating the probability of detecting the bacterium by culturing while maintaining the chance for real-time PCR to detect DNA from either dead or VBNC bacteria still present within the fish. The possible presence of both viable, dead and VBNC bacteria in variable frequencies in these fish would also explain the lack of direct correlation between culturing and GU counts as quantified by real-time PCR (Table 2).

The same seawater farm (no. 5) was sampled again approximately 3 months after antibiotic treatment, where *A. salmonicida* was detected in two of the four sampled fish by culturing, while real-time PCR detected *A. salmonicida* in all four fish and all 20 organs (Table 2). The question remains here whether the real-time PCR mostly detected DNA from dead bacteria or whether there were low amounts of viable *A. salmonicida* present in all fish and most of the organs, which were only detected in two fish (three organs) by culturing.

In the other seawater farm (no. 4), one of the two examined fish was found positive by culturing, while both fish were found positive by real-time PCR (Table 1). The organs where *A. salmoncida* was detected by real-time PCR were the spleen and intestine in both fish, along with the brain in one fish. Detection of *A. salmonicida* in the spleen and brain correlates with the findings of the bacterium by real-time PCR in fish from freshwater farms. However, it is interesting that the intestine was also found positive in both fish; in accordance, Hiney *et al.* (1994) stated that the intestine may be the primary location of *A. salmonicida* in salmon. Results from this seawater farm and the freshwater farms also bring attention to the importance of sampling from more than one or two organs in order to be sure of avoiding false negatives regarding the detection of *A. salmonicida* in fish.

In summary, our present findings indicate that there may be carrier fish harbouring VBNC *A. salmonicida* in Danish freshwater and seawater rainbow trout farms and that the spleen, brain and intestine could play an important role in *A. salmonicida* infection and persistence of VBNC. Further studies are needed for obtaining more knowledge about VBNC and carrier fish. The real-time PCR assay showed higher sensitivity for the detection of *A. salmonicida* than the culture method and exhibited a high reproducibility and efficiency. The real-time PCR assay presents a proficient tool for the detection of *A. salmonicida* in fish. One must keep in mind though that not all *A. salmonicida* seem to possess the target plasmid pAsal1. In order to be certain of avoiding false negatives, another sensitive detection method with a different target would need to be employed.

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Manuscript III

Epidemiology and genetics of *Aeromonas salmonicida* using whole genome sequencing

Epidemiology and genetics of *Aeromonas salmonicida* using whole genome sequencing

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Running title: Epidemiology of *Aeromonas salmonicida*

Abstract

Sea-reared rainbow trout production in Denmark currently struggles with furunculosis, a septicemic infection caused by the bacterium *Aeromonas salmonicida* subsp. *salmonicida*. Developing an effective control strategy is vital for future production, but this requires having knowledge of the epidemiology, as well as the genetic and virulent variability of the Danish *A. salmonicida* isolates. In order to obtain this, the genomes of 101 *A. salmonicida*, including 99 Danish isolates, one Scottish strain and the type strain NCIMB 1102, were sequenced using the Illumina HiSeq platform. Isolates were *de novo* assembled, examined for presence of plasmids, virulence and iron acquisition proteins and antibiotic resistance genes. Single Nucleotide Polymorphisms were aligned and subjected to Bayesian temporal phylogenetic and maximum likelihood tree reconstruction using the published genome of *A. salmonicida* A449 as reference. Bayesian temporal phylogenetic reconstruction suggests that four major introductions of *A. salmonicida* into Denmark have occurred. The introductions correlate with the freshwater and subsequent seawater expansion of rainbow trout production. Initial transmission of the bacterium could have been from seawater to freshwater or vice versa, both scenarios are open and most minor clades include a mixture of strains from different fresh- and seawater farms. Genetic variation of *A. salmonicida* is mostly associated with their plasmids and plasmid encoded virulence factors. Nine *A. salmonicida* harbored worldwide known antibiotic resistance genes against several antibiotics. These findings provide novel information regarding the Danish *A. salmonicida* population and demonstrate that whole genome sequencing is a highly useful tool for studying homogenous bacteria such as *A. salmonicida*.

Keywords: *Aeromonas salmonicida*, furunculosis, rainbow trout, whole genome sequencing, SNP analysis, BEAST, virulence factors

1. Introduction

Aeromonas salmonicida subsp. *salmonicida*, the causative agent of a septicemic infection furunculosis, was first described by Emmerich and Weibel (1894) at a German freshwater brown trout hatchery. Although the first rainbow trout (*Oncorhynchus mykiss*) hatchery in Denmark was already established in 1858 (Christensen, 1980), signs of furunculosis among fish were first described in the 1950s at freshwater rainbow trout farms (Rasmussen 1964). At this point the Danish freshwater rainbow trout production had begun its massive expansion. In the late 1970s, production was extended to seawater and dry pellet feed was introduced instead of the common wet feed consisting of herring, whiting, sand-eels and other marine fish not used for human consumption (Christensen, 1980). Both actions increased the growth of the Danish rainbow trout production even further.

Currently, it is in the expanded Danish sea-reared rainbow trout production that *A. salmonicida* is responsible for great financial losses. Despite fish being vaccinated before transfer from freshwater to seawater farms, furunculosis has occurred repeatedly during situations with elevated water temperatures (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000; Pedersen *et al.*, 2008). This situation, along with previous research, has led to the belief that *A. salmonicida* could be spread from freshwater to the sea by carrier fish that harbor the bacterium without showing any signs of disease (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000). Verifying this would be critical for developing an effective prevention strategy against furunculosis.

Several methods exist for molecular typing of bacterial isolates. The 'gold standard' for typing has long been Pulsed-field gel electrophoresis (PFGE), however, this method is time-consuming and lacks resolution power to distinguish the highly homogenous *A. salmonicida* on the subspecies level (Cunningham and Colquhoun, 2002; Beaz-Hidalgo *et al.*, 2008). Although other methods such as restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST) analysis can match the resolution of PFGE and can often provide results faster (Cunningham and Colquhoun, 2002; van Belkum *et al.*, 2007; Beaz-Hidalgo *et al.*, 2008) most of the methods are laborious and expensive (van Belkum *et al.* 2007). Recently a MLST-V based on eight fragments of housekeeping genes and four fragments of virulence associated genes was applied on 25 Danish isolates, though it did not have enough discriminatory power for distinguishing *A. salmonicida* at the subspecies level (authors' unpublished results).

Whole-genome sequencing (WGS) using next generation sequencing technology has over the past few years drastically decreased in cost and increased in speed, enabling its usage for studying everything from specific genes and virulence factors to epidemiology and long term evolution of various bacteria on a regular basis (Parkhill and Wren, 2011). Moreover, WGS provides the best overview of a studied population, since it avoids bias present in other molecular methods such as MLST, which only investigate a small part of the genome (Foxman *et al.* 2005; Parkhill and Wren, 2011).

In order to create an overview of the variation in genetics and virulence factors, as well as the epidemiology and evolution of Danish *A. salmonicida* isolates, a representative collection of 99 Danish *A. salmonicida* isolates varying in isolation years 1980 - 2014 and geographical regions, a Scottish strain and the type strain NCIMB 1102 were sequenced using the Illumina HiSeq platform. Sequences of all isolates were *de novo* assembled and analyzed using the published genome of *A. salmonicida* A449 (Reith *et al.*, 2008) as reference.

2. Methods

2.1 Bacterial isolates

Ninety-nine Danish *A. salmonicida* isolated from furunculosis outbreaks between1980 and 2014 were selected. The collection consisted of 42 *A. salmonicida* isolated from various freshwater farms, of which 40 were from rainbow trout and two from brown trout (*Salmo trutta*). Fifty-seven of the *A. salmonicida* were isolated from rainbow trout at various seawater farms, of which 14 isolates (isolated between 1981 - 2014) belonged to one large seawater farm and nine isolates (isolated between 1989 - 2010) to another large seawater farm named Sj4 and Sj3 respectively in this study. The Scottish *A. salmonicida* strain MT004 from Atlantic salmon (*Salmo salar* L.) is according to literature isolated around 1980. The *A. salmonicida* type strain NCIMB 1102 from England was isolated from an Atlantic salmon in year 1962. Extracted genomic DNA from all 101 *A. salmonicida* was used for sequencing.

2.2 Sample preparation

All *A. salmonicida* were grown in Veal Infusion Broth (VIB) (Difco) at 20^{o}C for 48 hours and then inoculated on blood agar plates (Colombia agar base (Oxoid) with 5% calf blood at 20^{o}C for 48 - 72 hours. Genomic DNA was extracted from bacterial colonies using a QIAGEN QIAamp DNA mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. DNA quality was determined by NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA) and DNA concentration by Qubit 2.0 fluorometer and Quant-iT dsDNA BR kit (Invitrogen, Carlsbad, CA, USA). All DNA extractions were immediately stored at -20^{0}C until further use.

2.3 Whole genome sequencing, de novo assembly, and antibiotic resistance genes

Genomic DNA was prepared for Illumina pair-end sequencing using the Illumina (Illumina, Inc., San Diego, CA) NexteraXT® Guide 150319425031942 following the protocol revision C (http://support.illumina.com/downloads/nextera_xt_sample_preparation_guide_15031942.html). A sample of the pooled NexteraXT Libraries was loaded onto an Illumina HiSeq reagent cartridge using HiSeq Reagent Kit v2 and 500 cycles with a Standard Flow Cell. The libraries were sequenced using an Illumina platform and HiSeq Control Software 2.3.0.3. All isolates were pair-end sequenced. Raw sequence data have been submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession no.: xxxxxxxx. The raw reads were *de novo*

assembled using the assemble pipeline (version 1.0) available from the Center for Genomic Epidemiology (CGE) https://cge.cbs.dtu.dk/services/Assembler/ which is based on the Velvet algorithms for *de novo* short reads assembly (Zerbino and Birney, 2008). Full genomic data can be retrieved from the supplementary information (SI) appendix, Table S1.

Identification of acquired antibiotic resistance genes (ARGs) was performed through assembled genomes using the pipeline ResFinder (version 2.1) (Zankari *et al.*, 2012) available from Center for Genomic Epidemiology (http://cge.cbs.dtu.dk/services/). Threshold for presence of an ARG in an isolate was set to 75% similarity expressed as percent sequence identity (ID) and 60% of alignment length (coverage) of resistance gene.

2.4 Single Nucleotide Polymorphisms (SNPs)

SNPs were determined using the pipeline; CSI phylogeny (Leekitcharoenphon *et al.*, 2012; Kaas *et al.*, 2014*)* available on the CGE (www.genomicepidemiology.org). In principle, the paired-end reads were mapped to the reference chromosome, the French *A. salmonicida* strain A449 isolated year 1975 from a brown trout (accession number CP000644, chromosome length 4,702,402 bp) using Burrows-Wheeler Aligner (BWA) version 0.7.2 (Li and Durbin, 2009). The 'mpileup' module in SAMTools version 0.1.18 (Li *et al.*, 2009) was used to identify SNPs. Qualified SNPs were determined when fulfilling the following criteria: 1) a minimum distance of 10 bps between each SNP, 2) a minimum of 10% of the relative depth at SNP positions, 3) the mapping quality was more above 25, 4) the SNP quality was more than 30 and 5) all indels were excluded. The SNPs from each genome were concatenated to a single alignment corresponding to position of the reference genome. The concatenated sequences were subjected to maximum likelihood tree using Fastree (Price *et al*., 2009)

2. 5 Temporal Bayesian Phylogenetic tree

SNPs were subjected to Bayesian temporal phylogenetic reconstruction using BEAST (Bayesian Evolutionary Analysis Sampling Trees) version 1.7 (Drummond and Rambaut, 2007; Drummond *et al.*, 2012) to estimate mutation rate and divergence time. Combinations of population size change and molecular clock were evaluated to identify the best-fit model (exponential clock and coalescent Bayesian skyline). The Bayesian temporal tree was constructed using the best-fit model. The BEAST MCMC chains were simulated for 300 million steps and subsampled every 10,000 steps. The final single maximum clade credibility (MCC) was examined using TreeAnnotator (Drummond *et al.*, 2012) with 10% of the MCMC steps discarded as burn-in. Statistical confidence was represented by the 95% highest posterior density (HPD) interval.

2.6 Virulence and iron acquisition proteins

To compare presence of virulence and iron acquisition proteins among all *A. salmonicida* isolates, a blastp search (Altschul *et al.*, 1990) was performed with 78 known virulence associated and iron

acquisition protein sequences (SI appendix, Table S2) found in the NCBI protein database against the assembled *A. salmonicida* genomes. Threshold limit for presence of protein in an isolate was set to 75% ID.

2.7 Plasmid profiles

The plasmid content of each *A. salmonicida* was analyzed by using blastn (Altschul *et al.*, 1990) with 11 known *A. salmonicida* plasmid sequences found in the NCBI database (SI appendix, Table S3) against the assembled *A. salmonicida* genomes. Threshold limit for presence of plasmid in an isolate was set to 75% ID and 60% coverage of plasmid due to the long length of plasmid sequences. Acquired ARGs present in the 11 plasmid sequences were determined using the pipeline ResFinder (version 2.1) (Zankari et al., 2012) with the above settings for threshold limit.

3. Results

3.1 Phylogeny

A total of 667 SNPs were identified in the chromosome from the *A. salmonicida* isolates. The French reference strain A449 displayed an average SNP difference of 147 to the rest of the isolates. The Scottish isolate and the type culture NCIMB 1102 had an average SNP difference of 115 and 41 respectively, while two Danish *A. salmonicida* isolated from brown trout (Mj2 1990 and Sd8 1992) had an average difference of 50 and 42 respectively. The average SNP difference among the Danish isolates was 47 and 46 SNPs between Danish isolates from freshwater versus isolates from seawater. The three Danish isolates with the highest average SNP difference were Sj7 1980 with 92 SNPs, Mj12 2014 with 67 SNPs and Mj4 2008 with 61 SNPs. Based on the alignment of the 667 SNPs, two trees were constructed: a Bayesian temporal tree (Fig. 1) with a Baysian Skyline population size change and an exponential clock rate as the best fit combination model for the *A. salmonicida* population and a maximum likelihood tree (SI appendix, Fig. S1) for topology confirmation. The two trees showed similar topology and the Bayesian tree (Figure 1) was illustrated with obtained genetic information regarding acquired ARGs, virulence and iron acquisition proteins and plasmid profiles of each *A. salmonicida* isolate for further analysis.

The mutation rate of *A. salmonicida* isolates was estimated to be 1.93×10^{-7} substitutions/site/year, which corresponds to 0.91 SNPs/genome/year. The most recent common ancestor of the *A. salmonicida* isolates was estimated to have emerged in ~1915 (95% HPD interval 1764 - 1947). There are two major clades originating back to ~1926 (95% HPD interval 1881 - 1950) that each branched out further into two more clades in ~1936 (95% HPD interval 1922 - 1958) and ~1970 (95% HPD interval 1934 - 1974) respectively, resulting in roughly four main introductions of *A. salmonicida* in Denmark: ~1973 (95% HPD interval 1958 - 1979), ~1973 (95% HPD interval 1964 - 1981), ~1948 (95% HPD interval 1934 - 1964) and ~ 1946 (95% HPD interval 1939 - 1961). From approximately 1975 - 1995 the Danish *A. salmonicida* population experienced a massive clonal expansion. There was a correlation of local geographical transmission among the Danish freshwater

isolates grouped together in the upper clade of the tree. There was another transmission link between isolates from a freshwater farm Mj10 and isolates from two seawater farms that had received fish from this farm.

3.2 Antibiotic resistance

All sequenced *A. salmonicida* isolates harbored three ARGs against beta-lactam antibiotics encoded on the chromosome (Table 1). Nine Danish *A. salmonicida* isolates also harbored several other plasmid encoded resistance genes against trimethoprim, sulphonamide and aminoglycoside antibiotics (Table 1). All three isolates from freshwater farm Mj10 sampled during different years harbored ARGs against several of the above mentioned antibiotics. The same ARGs against multiple antibiotics were found in isolates sampled from three seawater farms (Mj8 1997, Mj11 2014 and Mj3 2014) located in the same bay that all received fish from the freshwater farm Mj10. The French reference strain A449 also harbored ARGs against beta-lactam, sulphonamide, aminoglycoside, phenicol and tetracycline antibiotics and more resistance genes are described by (Reith *et al*., 2008).

3.3 Virulence and iron acquisition

Out of 78 investigated protein sequences, 22 were considered as absent (<65% ID) in one or more isolates (Fig. 2). The Type Three Secretion System (T3SS) Effector protein AopP encoded on plasmid pAsal1 by the *aopP* gene was absent in 50% the *A. salmonicida* isolates, including the reference strain A449. A cluster of 15 T3SS related proteins were absent in 25 isolates. In nine of the 25 isolates, the T3SS putative tyrosine phosphatase AopH and its chaperone that are encoded on pAsa5 and have homologs encoded on pAsa6 were also absent. Three of the isolates were also missing the T3SS putative serine/threonine kinase AopO and its chaperone that are encoded on pAsa5, while isolate Mj12 2014 was missing the extracellular nuclease protein (48% ID) coded by the gene *nucH* on the chromosome. Isolate Sj4 1998, which is not included in above mentioned group of 25 isolates, did not possess the tetragonal surface virulence array protein VapA (A-layer) encoded on the chromosome. Lastly, the chromosome encoded ABC-type ferric siderophore transporter permease protein only showed 75% ID in all sequenced *A. salmonicida* as well as the reference strain A449.

3.4 Plasmid profiles

All examined *A. salmonicida* isolates displayed presence of multiple plasmids (SI appendix, Table S4). Seven plasmids were present in one or more isolates, while four plasmids: pAr-32, pRAS1, pRAS3.1 and pRAS3.2 were not present in any isolates. The only plasmid found in all isolates was pAsa1, although pAsa2 showed high stability with a presence of 99% among the isolates. Plasmids pAsa5 and pAsa6 were present in 90% and 85% respectively. The two plasmids pAsa3 and pAsal1were present in 76% and 52% of the isolates respectively, while pAsa4 was only present in the reference strain A449. Twelve different plasmid profiles were detected among the isolates, with

one profile consisting of pAsa1, pAsa2, pAsa3, pAsa5, pAsa6 and pAsal1 representing 44% of the isolates (Table 2).

Five plasmids are known for harboring ARGs (R plasmids): pAsa4, pAr-32, pRAS1, pRAS3.1 and pRAS3.2 (SI appendix Table S3) and only plasmid pAsa4 was present in any of the investigated *A. salmonicida* (the reference strain A449).

All 51 *A. salmonicida* isolates in which the AopP protein encoded on pAsal1 was absent (Fig. 2), were missing plasmid pAsal1 (SI appendix Table S4). Of the 25 *A. salmonicida* isolates that were missing a cluster of 15 T3SS proteins encoded on pAsa5, ten were also missing the plasmid pAsa5. The remaining 15 isolates all displayed <80% coverage of the pAsa5. All nine *A. salmonicida* isolates that lacked the protein AopH and its chaperone that are encoded on pAsa5 and have homologs on pAsa6 showed <80% coverage for pAsa5 and were missing pAsa6.

4. Discussion

4.1 Phylogeny

A. salmonicida subspecies is known to be a highly homogenous group that is considered clonal (Wiklund and Dalsgaard, 1998; Garcia *et al.*, 2000; Cunningham and Colquhoun, 2002; Beaz-Hidalgo *et al.*, 2008). The fact that only a total of 667 SNPs were found in the entire 4,702,402 bp long chromosome among the investigated *A. salmonicida* varying in year of isolation (span of 34 years), geographical region, and host fish species only confirms this further. The highest average SNP difference was found between the French reference strain A449 and the rest of the *A. salmonicida* isolates (average of 147 SNPs), which is not a large difference when considering that A449 was isolated in France and from a brown trout, while almost all (97out of 99) of the Danish isolates were isolated from rainbow trout. When comparing the average SNP difference between the Scottish strain from Atlantic salmon and the rest of the isolates, the results were even lower (average of 115 SNPs). The two Danish *A. salmonicida* isolated from brown trout (Mj2 1990 and Sd8 1992) and the type strain NCIMB 1102 from Atlantic salmon also grouped together with Danish *A. salmonicida* from rainbow trout in one of the four major clades (Fig. 1) and only have an average of 41, 50 and 52 SNP difference, which challenges the theory of *A. salmonicida* genome adapting to the environment of their specific hosts species (Reith *et al.*, 2008). More *A. salmonicida* isolates from various fish species would, however, need to be sequenced in order to shed more light on this theory.

Noticeably there appears to be four major *A. salmonicida* introductions to Denmark, giving rise to four major clades (Fig. 1). The two introductions that occurred in ~1973 (95% HPD interval 1958 - 1979) and ~1973 (95% HPD interval 1964 - 1981) and gave rise to the two upper clades (Fig. 1), seemingly took place right before the massive clonal expansion during 1975 - 1995, which all four clades underwent. The two introduction points in 1973 and the expansion period of all four clades correspond to the time period where rainbow trout farming in Denmark began expanding out to

seawater and intensifying their production. The two bottom clades were introduced further in the past ~1948 (95% HPD interval 1934 - 1964) and ~ 1946 (95% HPD interval 1939 - 1961) respectively and also contain *A. salmonicida* with older isolation years (average year of isolation 1991), than the two upper clades that include *A. salmonicida* with an average isolation year of 2001 (Fig. 1).

The introduction of the two bottom clades correlate with the end of the Second World War and the beginning of an expansion of rainbow trout production in Danish freshwater. When examining the branches of each of the four major clades, there is a possibility that *A. salmonicida* might have been introduced into Denmark through seawater and was from thereon spread to freshwater. One explanation for this possible scenario is the fact that wet feed, comprised of marine fish including sand-eels, was used at all Danish fish farms until the late 1970s (Christensen, 1980). *A. salmonicida*, although the atypical type, has been isolated from sand-eels caught in the surrounding seawater of Denmark (Dalsgaard and Paulsen, 1986). It is also known that *A. salmonicida* can be harbored by various farmed and wild freshwater and seawater aquatic animals (Bernoth *et al.*, 1997). It could be thus be hypothesized that the wet feed could be the cause behind a possible initial transmission of the bacterium from seawater to freshwater. However, the bacterium could also have been transmitted from freshwater to seawater, which is the widespread theory i.e. that *A. salmonicida* is present in freshwater fish showing no signs of diseases (carriers) and are then transferred out to seawater with the fish, where outbreaks occur during high temperatures (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000; Pedersen *et al*., 2008).

The local transmission pattern of *A. salmonicida* among the Danish farms also suggests that transmission of isolates from freshwater to seawater farms have occurred, as exemplified by a minor clade where ARGs were transmitted from a freshwater farm to seawater farms, though this will be discussed under the antibiotic resistance section. Isolates from different freshwater farms are moreover mixed with different isolates from seawater in most of the minor clades. Though, in general it is hard to find specific geographical correlations between the fish farms. One of the main causes for this could be the widespread trade of fingerlings for anglers in Denmark throughout the years as well as local trade among fish farmers. There is nonetheless a correlation among the group of freshwater farms isolates in the top clade. Mj13 is located upstream to Mj16 in a stream that runs out into a river named Guden Å. Two other farms (Mj12 and Mj2) are also located at streams that lead out to Guden Å and one of these (Mj12) produces brown trout.

Finally, it was also found that isolates grouped in the two bottom clades were missing a higher amount of virulence associated proteins (average of 1.0 protein per isolate), compared to isolates grouped in the two upper clades where the average absence of virulence associated proteins was 0.6 per isolate (Fig. 1). This indicates that the most recently common ancestor of the bottom two clades from ~1936 (95% HPD interval 1922 - 1958), presumably harbored itself and gave rise to two older lineages that harbored fewer virulence associated proteins than the more recently introduced two lineages (the two upper clades in the phylogeny tree). Considering these results, it could be

suggested that the upper two clades might consist of more virulent *A. salmonicida* that were introduced from a more recent and more virulent ancestor around 1970.

4.2 Antibiotic resistance

Interestingly, all investigated *A. salmonicida* isolates possessed three beta-lactam ARGs. Since the genes are encoded on the chromosome, it seems that either they have always been a part of the *A. salmonicida* genome repertoire, or they must have been acquired at least around 67 - 250 years ago. Nine Danish *A. salmonicida* also harbored resistance genes against trimethoprim, sulphonamide and aminoglycoside antibiotics, which are all plasmid encoded. Trimethoprim and sulphonamide are also two of the scarce number of antibiotics allowed to be used for treatment of bacterial diseases in Danish fish farms. All three *A. salmonicida* isolates from freshwater farm Mj10, isolated year 1982, 2009 and 2010 harbored resistance genes against at least two of the above mentioned antibiotics (Table 1). Fish from farm Mj10 have always been transferred out to a bay, where several seawater farms are located. In *A. salmonicida* isolated during 2014 from two of these seawater farms, the same set of resistance genes were detected as those seen in *A. salmonicida* from the freshwater farm Mj10 during 2009 and 2010. Fittingly, the two freshwater isolates form a minor clade with the two seawater isolates in the Bayesian temporal tree, according to which the isolates spread from the freshwater to seawater (Fig. 1). In seawater farm Mj8, which is also located in the bay, an *A. salmonicida* isolate from 1997 did harbor ARGs against the mentioned antibiotics as well. However, these ARGs were slightly different than those seen in the Mj10 1982 isolate, where one otherwise would expect the resistance genes had originated (Table1). This could be associated with the fact that trimethoprim was not licensed for use in Denmark until 1983 and the isolate from 1982 had therefore not acquired ARGs against this antibiotic.

None of the nine Danish isolates harbored any of the five investigated R plasmids, though eight did show coverage (< 60%) of at least one of the R plasmids (SI appendix, Table S4), indicating they could have acquired ARGs from the plasmids in the past through horizontal gene transfer and then lost the plasmid. Isolate Sj4 2014 that showed zero coverage of all R plasmids harbored ARGs *strA* and *strB*, which were present in R plasmid pRAS2 isolated from *A. salmonicida* in salmon from Norway (L'Abée-Lund and Sørum, 2000). This plasmid was not included in the analysis, since only a couple of gene sequences from this plasmid are available in Genbank. However, pRAS2 could be or have been present among the Danish *A. salmonicida* population. Noteworthy, the only other isolate harboring ARGs *strA* and *strB* was isolated in 2011 from farm Nj1, though there has not been any transfer of fish from this farm to Sj4, whereby the two occurrences of the ARGs *strA* and *strB* are incidental. When looking at the broader picture, there are many highly similar broad host R plasmids that have been isolated from *Aeromonas* species in various environments all over the world that harbor the same ARGs found in the Danish *A. salmonicida* (L'Abée-Lund and Sørum, 2001; Sørum *et al.*, 2003; Kadlec *et al.*, 2011; Muziasari *et al.*, 2014). Present findings only provide further evidence of this widespread dissemination of R plasmids and ARGs, although the prevalence of these ARGs seems to be similar (9%) to the low prevalence of 5% found by Dalsgaard *et al.*, (1994).

4.3 Iron acquisition

Iron acquisition has been proven to be an important factor for virulence in almost all bacterial pathogens, including *A. salmonicida* where it also seems to be linked to survival in aquatic environments (Reith *et al.,* 2008). Iron is also thought to be linked to the innate immune response in the host, which in turn attempts limiting iron availability in order to lower virulence and access of the pathogen into the host (Ganz, 2009; Lee *et al.,* 2014). Due to the important nature of iron acquisition and the fact that all the investigated proteins related to iron acquisition were encoded on the chromosome, it was expected that all 102 *A. salmonicida* isolates possessed all the investigated iron acquisition proteins (SI appendix, Table S2). Though, it has to be noted that the ABC-type ferric siderophore transporter permease protein only showed 75% ID in all *A. salmonicida* isolates.

4.4 Virulence

In addition to iron acquisition proteins, numerous of potential virulence factors have been identified in *A. salmonicida*, including extracellular proteases, lipases, adhesins, and functional secretion systems (Burr *et al*., 2002; Rasch *et al.*, 2007; Reith et al., 2008; Dallaire-Dufresne *et al*., 2014). The majority of potential virulence proteins investigated in this study was present in all sequenced *A. salmonicida* and the reference strain. Though, two proteins encoded on the chromosome and 20 encoded on plasmids were missing in at least one isolate (SI appendix, Table S2). In agreement with the present results, DNA micro array study of virulence genes in *Aeromonas* species including several subspecies *salmonicida* isolates by (Nash *et al*., 2006) also showed high degree of variability among genes associated with plasmids, whereas genes encoded on the chromosome did not vary significantly.

All of the above mentioned 20 plasmid encoded proteins were related to the functional type three secretion system (T3SS). This secretion system is wide spread among Gram-negative bacteria and has several functions, including: disrupting host cells by translocating toxins (effector proteins) into their cytoplasm, preventing phagocytosis by leukocytes, and establishing systemic infection (Burr *et al*., 2003; Stuber *et al*., 2003; Burr *et al*., 2005; Dacanay *et al.,* 2006; Rasch *et al.,* 2007; Dallaire-Dufresne *et al*., 2014). T3SS is also the only virulence factor proven to be essential for virulence of *A. salmonicida*, as all *in vitro* and *in vivo* studies involving inactivation of T3SS structural proteins in *A. salmonicida* strains have resulted in non-virulent *A. salmonicida* mutants (Burr *et al.,* 2002; Burr *et al.*, 2003; Stuber et al., 2003; Burr *et al.*, 2005; Dacanay *et al.*, 2006; Froquet *et al*., 2007). Nevertheless, among the 20 missing T3SS related proteins in this study were T3SS structural proteins, while all the *A. salmonicida* in this study are isolated from furunculosis outbreaks, whereby one would assume that all the *A. salmonicida* isolates are virulent.

There are 36 T3SS encoding genes located on the large plasmid pAsa5 (Reith *et al.*, 2008; Najimi *et al.,* 2009: Tanaka *et al.*, 2012; Vincent *et al.*, 2016) and 19 of them that were investigated in this study were missing in at least three isolates (SI appendix, Table S2). Initially these 36 genes were

found to be located on a 140 kb plasmid named pASvirA (Stuber *et al.*, 2003) and while pASvirA and pAsa5 are almost the same size, it remains unclear whether they are variants of the same plasmid (Najimi *et al.*, 2009). Though, both plasmids become unstable under stressful conditions like being subjected to growth in temperature above 22 - 25^{o}C (Stuber *et al.*, 2003; Tanaka *et al.*, 2012; Dallaire-Dufresne *et al.*, 2014). While pASvirA is seemingly lost by *A. salmonicida* during the stressful conditions (Stuber *et al.*, 2003), pAsa5 is thought to undergo genetic rearrangement resulting in the loss of its T3SS region caused by activation of IS*AS11*insertion sequence (IS) elements (Tanaka *et al.*, 2012). This could explain the fact that all the 25 *A. salmonicida* isolates missing the cluster of T3SS proteins, encoded on pAsa5 in our study still harbored the plasmid, but displayed <80% coverage. The only issue with this justification is that all *A. salmonicida* cultures in our laboratory are always grown at 20^{o}C, meaning it is unlikely that growth at high temperature triggered the activation of IS*AS11*. It is unclear what other cause for the rearrangement of pAsa5 could have been.

Plasmid pAsa6 also shares most of its sequences, including sequences for AopH and its chaperone, with pAsa5 with a close to 100% nucleotide sequence similarity (Najimi *et al.*, 2009). In agreement, all nine *A. salmonicida* isolates in our study that lacked AopH and its chaperone showed <80% coverage of pAsa5 and were missing pAsa6 (SI appendix, Table S4). Presence of AopH and chaperone homologs on pAsa6 would also explain why the sequences for these two proteins were not missing in all of the above mentioned 25 isolates i.e. the two protein sequences in all but nine isolates could still be present on pAsa6. Though, it remains unclear why only three of the 25 isolates were also missing the AopO protein and its chaperone, since both proteins are only encoded on pAsa5. One reason could be that they were encoded in a different region and were thus not rearranged along with the other 15 T3SS protein sequences.

Unlike most of the T3SS proteins, the AopP protein is encoded on plasmid pAsal1 and the protein was missing in 50% of the *A. salmonicida* isolates investigated in this study (Fig. 2). Interestingly, pAsal1 was present in isolate Sj 1981 and the Scottish isolate, both of which did not harbor the AopP protein. The isolates did possess the nucleotide sequence for the *aopP* gene; however, both sequences had an identical frameshift mutation caused by point deletions (data not showed) that presumably lead to an incorrect translation of the AopP protein sequence. Apart from the two mentioned isolates, all *A. salmonicida* that were missing the AopP protein sequence were also missing the plasmid pAsal1. Previous studies have shown that pAsal1 is lost due to activation of the same insertion sequence as in pAsa5 (Daher *et al.* 2011; Tanaka *et al* 2012; Attéré *et al.*, 2015; Vincent *et al.*, 2016), though there was no correlation between a lowered coverage of pAsa5 and absence of pAsal1. Though the plasticity of *A. salmonicida* pAsal1 is complex, as the precise mechanism responsible for loss of pAsal1 remains unknown (Attéré *et al.*, 2015) and at least three larger variants of the plasmid exist: pAsal1B, pAsal1C and pAsal1D (Trudel *et al.*, 2013; Attéré *et al.*, 2015). All the variants harbor another insertion sequence element called IS*AS5* that in pAsal1C and pAsal1D disrupts the IS*AS11*, which hence cannot be activated leading to the prevention of loss of these two variants of pAsal1 during stressful conditions (Attéré *et al.*, 2015). Although the IS*AS5*

457 does not disrupt IS*AS11* in pAsal1B, according to Attéré *et al.*, (2015) it could still prevent
458 activation of IS*AS11*.
459
460 The last two missing virulence associated proteins, the A-layer protein encoded by the *vapA* gene
461 and the extracellular nuclease protein encoded by the gene *nucH*, were missing in isolate Sj4 1998
462 and Mj12 2014 respectively. The absence of the extracellular protein in isolate Mj12 2014
463 correlates with its unusually high average SNP difference of 67 compared to other Danish *A.*
464 *salmonicida*. Isolate Sj4 1998 that is missing *vapA* does not have a higher SNP difference (47)
465 compared to the other Danish *A. salmonicida*. Interestingly, isolate Sj7 1980 that has the highest
466 average SNP difference of 92 SNPs among the Danish isolates, had an ID percentage of 87% for
467 *vapA* and when the isolates' *vapA* gene sequence was manually investigated with the program
468 BioEdit (Hall, 1999), it was discovered that the first half of the nucleotide gene sequence differs
469 significantly from the *vapA* nucleotide sequences of the other *A. salmonicida* isolates (data not
470 shown). As illustrated on Fig. 2, there was otherwise an overall high similarity among all isolates
471 regarding all the chromosome encoded virulence associated protein sequences. The high prevalence
472 and similarity of the A-layer (VapA) in *A. salmonicida* discovered in this study, along with previous
473 findings of *A. salmonicida* surface structures in contact with host defenses having a high antigenic
474 conservation (Chart *et al.*, 1984), could also provide valuable knowledge for future vaccine
475 development.
476
477 **4.5 Plasmid profiles**
478
479 Out of the twelve plasmid profiles found in this study, the most abundant profile consisting of
480 pAsa1, pAsa2, pAsa3, pAsa5, pAsa6 and pAsal1 represented 44% of the *A. salmonicida* isolates
481 (Table 2). In the study by Nielsen *et al.* (1993) of *A. salmonicida* from various geographical
482 locations using DNA restriction fragment plasmid profiling, a plasmid profile with the following
483 five plasmids: pAsa1, pAsa2, pAsa3, pAsa5 and pAsal1was the most common profile among
484 Danish *A. salmonicida* isolates, representing 32% of the 57 investigated Danish strains in that study.
485 Nielsen *et al.* (1993) also investigated *A. salmonicida* NCIMB 1102, which belonged to the plasmid
486 profile group mentioned above and was thus missing pAsa6. Seemingly pAsa6 (molecular weight
487 of 18.5 kb) was not found in any of the 124 *A. salmonicida* strains investigated by Nielsen *et al.*
488 (1993). On the contrary, present results showed that pAsa6 was present in 87 *A. salmonicida*
489 including NCIMB 1102. Possible explanations for the disagreeing results regarding pAsa6 could be
490 that pAsa6 was not observed on gel by Nielsen *et al.* (1993) due being present in a low copy
491 number, or pAsa6 could integrate into the *A. salmonicida* chromosome due to the abundance of IS
492 elements within the plasmid (Najimi *et al*., 2009).
493
494 Present findings support previous results by Nielsen *et al.* (1993), Boyd *et al.* (2003) and Attéré *et*
495 *al.* (2015) regarding high stability of plasmid pAsa1 and pAsa2 and instability of plasmid pAsa3
496 and pAsal1. Attéré *et al.* (2015) suggested an explanation for the stability of pAsa1 and instability
497 of pAsal1 could be that pAsa1 and pAsa3 encode genes for a type II toxin-antitoxin (TA) system
498 that kills all daughter cells that do not receive the plasmids (Boyd et al., 2003), while the TA system

has not been found in plasmids pAsa2 and pAsal1 (Shao *et al.*, 2011). Though there is still the issue regarding stability of pAsa2 that does not encode a TA system and the instability of pAsa3 that does have a TA system (Attéré *et al.*, 2015); an issue accentuated by the present findings. It might be possible that some clonal lineages do not acquire pAsa3 and pAsal1 (Attéré *et al.*, 2015), which cannot be ruled out according to our results, where out of 24 *A. salmonicida* that did not harbor pAsa3 and pAsal1 (plasmid profiles nine and ten), 17 clustered together in four minor clades (Fig. 1, four minor clades with a red ring).

5. Conclusion

The present findings have provided novel insight into the epidemiology of the disease causing Danish *A. salmonicida*, revealing four main introductions in consistency with the historical expansion of the Danish aquaculture production that could have been transmitted either from freshwater to seawater or vice versa. There was also transmission of isolates harboring ARGs from a freshwater farm to seawater farms, supporting the theory of *A. salmonicida* being spread from freshwater to seawater via carrier fish. The mixture of freshwater and seawater isolates from different farms in almost every minor clade and the lack of geographical connections among farms also indicates that the widespread trade of fingerlings and other fish could have played a role in the local dissemination of *A. salmonicida* in Denmark. The genome based analysis moreover showed genetic and virulence variability among the highly homogenous *A. salmonicida* population in Denmark, which consisted of isolates with varying plasmid profiles and plasmid encoded virulence proteins, especially those related to T3SS. Overall, WGS proved to be a highly useful tool for investigating Danish *A. salmonicida* and presented important new information about this bacterium.

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6. Contributions

Conceived and designed the experiments: ID, FMA. Contributed reagents/materials/analysis tools: SB, PL, ID, FMA. Performed field sampling: ID. Performed the experiments: SB, ID. Analyzed the data: SB, PL, ID, FMA. Wrote the paper: SB, PL. Critically revised the paper: FMA, ID.

7. References

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8. Tables

Table 1. Overview of acquired antibiotic resistance genes among the 101 *A. salmonicida* sequenced isolates and the reference A449. Threshold for presence of a resistance gene in an isolates was set to 75% similarity expressed as percent sequence identity (ID) and 60% of alignment length (coverage) of the resistance gene. Isolates are labeled according to region and year of isolation as in Figure 1.

A. salmonicida isolate	Beta-lactam			Trimethoprim		Sulphonamide		Aminoglycoside				Phenicol	Tetracycline
Reference A449 (France 1975)	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	*sul1*	-	-	-	*aadA1*	-	*cat*	*tet(E)*
Sd5 2005	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Mj10 2009	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Mj10 2010	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Nj1 2011	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	*strB*	*aadA1*	*strA*	-	-
Mj11 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Mj3 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Sj4 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	*dfrA14*	-	-	*sul2*	-	*strB*	-	*strA*	-	-
Mj10 1982	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	*sul1*	-	*aadA2*	-	-	-	-	-
Mj8 1997	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	*dfrA1*	*sul1*	-	-	-	*aadA1*	-	-	-
Nj2 2001	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj22 2002	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Nj4 2002	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj11 2003	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 2003	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd1 2004	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd4 2004	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 2004	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj7 2008	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj16 2008	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj4 2008	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 2008	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (a) 2009	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 (a) 2009	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 (b) 2009	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (b) 2009	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 2010	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (a) 2010	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (b) 2010	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 2011	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj9 2012	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj12 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd6 (a) 2013	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd6 (b) 2013	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj14 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd4 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd10 2014	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj7 1980	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj24 1980	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj18 1981	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj7 1981	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1981	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj16 1981	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd3 1982	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1982	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd2 1982	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1983	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj6 1983	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj15 1983	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd2 1983	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj7 1984	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj15 1984	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Nj3 1984	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj16 1985	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj5 1986	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj18 1986	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj16 1986	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj11 1987	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj16 1987	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj13 1987	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj5 1988	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1988	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-

Sd9 1988	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj11 1988	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj1 1989	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 1989	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj2 1990	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 1990	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj20 1990	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj5 1990	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Scotland	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj3 1991	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Nj5 1991	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj5 1991	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj18 1991	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1992	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj1 1992	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd8 1992	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj6 (a) 1993	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj6 (b) 1993	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj2 (a) 1993	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj2 (b) 1993	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj21 1993	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj19 1994	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj5 1994	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Nj3 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj12 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj1 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj4 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj5 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd2 1995	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj23 (a) 1996	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj23 (b) 1996	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj6 (a) 1996	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj6 (b) 1996	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sd7 1997	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj20 1997	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 1998	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (a) 1999	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Mj17 1999	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
Sj4 (b) 1999	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-
NCIMB 1102 (Type strain 1962)	*blaFOX-2*	*ampS*	*blaCEPH-A3*	-	-	-	-	-	-	-	-	-	-

Table 2. Overview of plasmid profiles among the 101 *A. salmonicida* sequenced isolates and the reference A449. Plasmid profile number is displayed to the right, as well as the number of *A. salmonicida* that have the respective profile. Presence and absence of a plasmid for the given profile is presented with a plus (present) and minus (absent) sign respectively, below each plasmid name.

Profile No.	No. of *A. salmonicida* isolates	Plasmids										
		pAsa1	pAsa2	pAsa3	pAsa4	pAsa5	pAsa6	pAsal1	pAr_32	pRAS1	pRAS3.1	pRAS3.2
1	45	+	+	+	-	+	+	+	-	-	-	-
2	1	+	-	+	-	+	+	+	-	-	-	-
3	2	+	+	+	-	-	+	+	-	-	-	-
4	4	+	+	+	-	-	-	+	-	-	-	-
5	1	+	+	+	-	+	-	+	-	-	-	-
6	3	+	+	+	-	-	-	-	-	-	-	-
7	1	+	+	+	-	-	+	-	-	-	-	-
8	4	+	+	+	-	+	-	-	-	-	-	-
9	2	+	+	-	-	+	-	-	-	-	-	-
10	22	+	+	-	-	+	+	-	-	-	-	-
11	16	+	+	+	-	+	+	-	-	-	-	-
12	1	+	+	+	+	+	-	-	-	-	-	-

9. Figure legends

Figure 1. Phylogeny of *A. salmonicida.* Bayesian temporal phylogenetic tree based on the alignment of 667 SNPs found among the 101 *A. salmonicida* sequenced isolates and the reference A449. The tree shows the most recent common ancestor of the *A. salmonicida* isolates dates back to ~1915 (95% HPD interval 1764 - 1947) and that there have been four main introductions of *A. salmonicida* in Denmark: ~1973 (95% HPD interval 1958 -1979), ~1973 (95% HPD interval 1964 - 1981), ~1948 (95% HPD interval 1934 - 1964) and ~ 1946 (95% HPD interval 1939 - 1961). The four main clades are each shaded with a color SNP differences between major clades are shown above the estimated year of emergence. The three non-Danish *A. salmonicida* each have their own color and have the following labels: Scotland, NCIMB 1102 (type strain 1962), Reference A449 (France 1975). The Danish isolates either have a black color (freshwater farms) or a blue color (seawater farms) and they are labeled by region of origin followed by year of isolation. Following abbreviations are used for regions in Denmark: Nj = Northern Jutland, Mj = Central Jutland, Sd = Southern Denmark, Sj = Zealand. A heatmap illustration with information regarding acquired ARGs, virulence and iron acquisition proteins and plasmid profile numbers of each *A. salmonicida* isolate is shown to the right of the tree. Presence and absence of protein sequences are illustrated by presence and absence of a red square, respectively. Plasmid profile number is shown and isolates that harbor ARGs against multiple antibiotics are labeled with "res". Four minor clades marked with a red ring consist solely of isolates without plasmid pAsa3 and pAsal1.

Figure 2. Heatmap illustrating presence and absence of 78 virulence associated and iron acquisition protein sequences found in the NCBI protein database among the 101 *A. salmonicida* sequenced isolates and the reference A449. Isolates are displayed on the right and sequence protein names on the bottom. Threshold limit for presence of protein in an isolate was set to 75% similarity, expressed as percent sequence identity (ID). Red color represents > 95% ID, pink color > 85% ID, dark blue > 75% ID and light blue > 65% ID.

10. Supplementary information appendix

Table S1. Full genomic data of the 101 sequenced *A. salmonicida* isolates. Isolates are labeled as stated in Figure 1.

Table S2. Overview of 78 virulence associated and iron acquisition protein sequences found in the NCBI protein database. The following is shown in the table: name of protein sequence, short description, location of the protein coding gene, Genbank accession number and the number of *A. salmonicida* isolates harboring the given protein sequence. Isolates are labeled as stated in Figure 1.

Table S3. Overview of 11 *A. salmonicida* plasmids found in the NCBI nucleotide database. The following is shown in the table: name of plasmid, short description, antibiotic resistance genes encoded on plasmid, length of plasmid (in base pairs), and Genbank accession number. Isolates are labeled as stated in Figure 1.

Table S4. Plasmid content of the 101 *A. salmonicida* sequenced isolates and the reference A449. Numbers under each plasmid name represent percent coverage of that plasmid (in base pairs) for a given isolate. If percent coverage is higher than 60%, the plasmid is said to be present in the isolate and the color of the cell is green. Threshold limit for presence of plasmid in an isolate was set to 75% similarity expressed as percent sequence identity (ID) and 60% of alignment length (coverage) of the plasmid. Following abbreviations are used for percent coverage: 100 = 100% coverage, <100 = from 80% up to 99% coverage, <80 = from 60% up to 79% coverage, <60 = from 10 up to 59% coverage, <10 = from 1% up to 9% coverage, 0 = 0% coverage. Isolates are labeled as stated in Figure 1.

Figure S1. Phylogeny of *A. salmonicida.* Maximum likelihood tree based on the alignment of 667 SNPs found among the 101 *A. salmonicida* sequenced isolates and the reference A449. Isolates are labeled as stated in Figure 1.

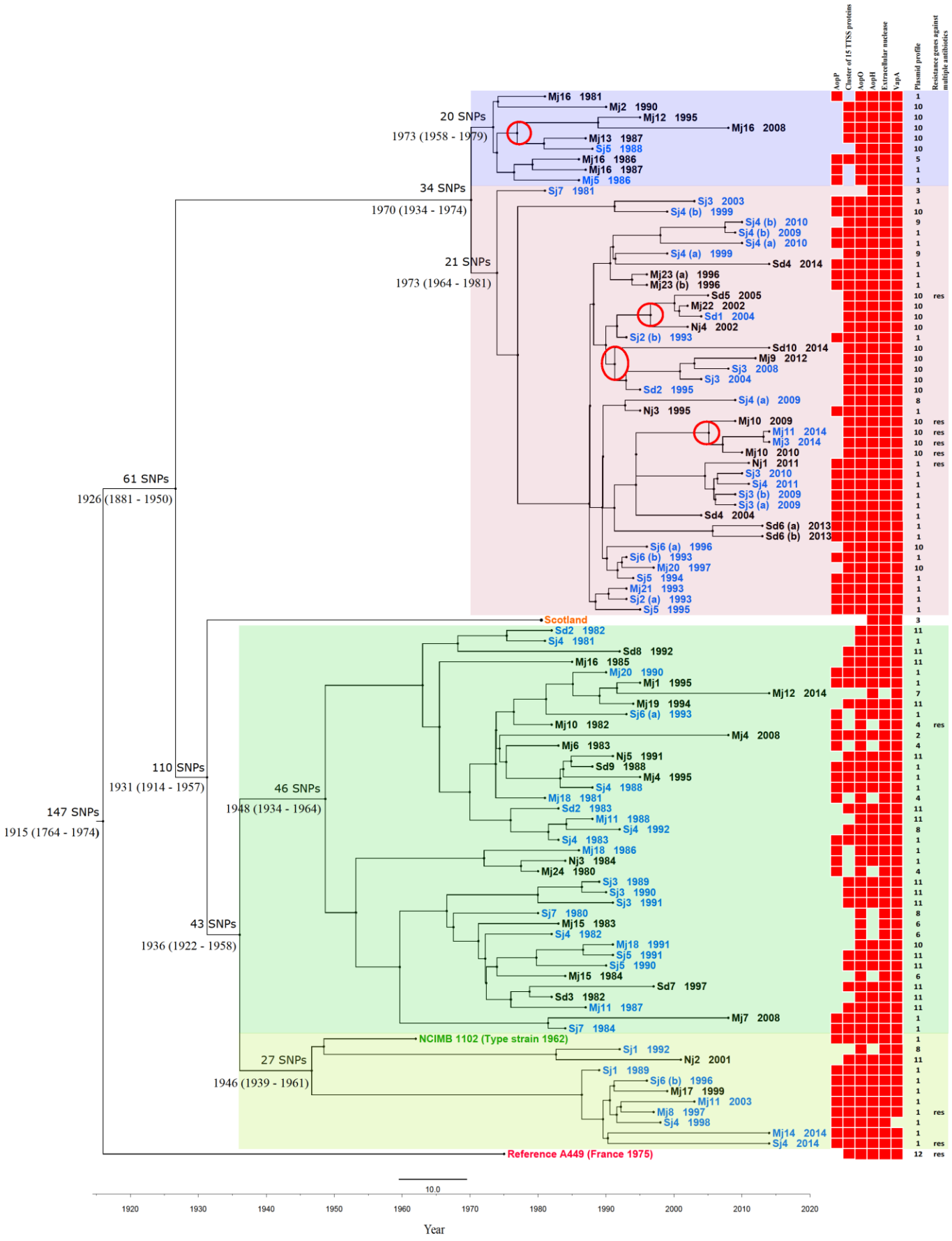
AopP
Cluster of 15 T3SS proteins
AopO
AopH
Extracellular nuclease
VapA
Plasmid profile
Resistance genes against multiple antibiotics
Mj16 1981
Mj2 1990
Mj12 1995
Mj16 2008
Mj13 1987
Sj5 1988
Mj16 1986
Mj16 1987
Mj5 1986
20 SNPs
1973 (1958 - 1979)
34 SNPs
1970 (1934 - 1974)
Sj7 1981
Sj3 2003
Sj4 (b) 1999
Sj4 (b) 2010
Sj4 (b) 2009
Sj4 (a) 2010
Sj4 (a) 1999
Sd4 2014
21 SNPs
1973 (1964 - 1981)
Mj23 (a) 1996
Mj23 (b) 1996
Sd5 2005
Mj22 2002
Sd1 2004
Nj4 2002
Sj2 (b) 1993
Sd10 2014
Mj9 2012
Sj3 2008
Sj3 2004
Sd2 1995
Sj4 (a) 2009
Nj3 1995
Mj10 2009
Mj11 2014
Mj3 2014
Mj10 2010
Nj1 2011
Sj3 2010
Sj4 2011
Sj3 (b) 2009
Sj3 (a) 2009
Sd4 2004
Sd6 (a) 2013
Sd6 (b) 2013
Sj6 (a) 1996
Sj6 (b) 1993
Mj20 1997
Sj5 1994
Mj21 1993
Sj2 (a) 1993
Sj5 1995
61 SNPs
1926 (1881 - 1950)
Scotland
Sd2 1982
Sj4 1981
Sd8 1992
Mj16 1985
Mj20 1990
Mj1 1995
Mj12 2014
Mj19 1994
Sj6 (a) 1993
Mj10 1982
Mj4 2008
Mj6 1983
Nj5 1991
Sd9 1988
Mj4 1995
Sj4 1988
Mj18 1981
Sd2 1983
Mj11 1988
Sj4 1992
Sj4 1983
Mj18 1986
Nj3 1984
Mj24 1980
Sj3 1989
Sj3 1990
Sj3 1991
Sj7 1980
Mj15 1983
Sj4 1982
Mj18 1991
Sj5 1991
Sj5 1990
Mj15 1984
Sd7 1997
Sd3 1982
Mj11 1987
Mj7 2008
Sj7 1984
110 SNPs
1931 (1914 - 1957)
46 SNPs
1948 (1934 - 1964)
147 SNPs
1915 (1764 - 1974)
43 SNPs
1936 (1922 - 1958)
NCIMB 1102 (Type strain 1962)
Sj1 1992
Nj2 2001
27 SNPs
1946 (1939 - 1961)
Sj1 1989
Sj6 (b) 1996
Mj17 1999
Mj11 2003
Mj8 1997
Sj4 1998
Mj14 2014
Sj4 2014
Reference A449 (France 1975)
10.0
1920
1930
1940
1950
1960
1970
1980
1990
2000
2010
2020
Year

Figure 1. Phylogeny of *A. salmonicida.* Bayesian temporal phylogenetic tree based on the alignment of 667 SNPs found among the 101 *A. salmonicida* sequenced isolates and the reference A449. The tree shows the most recent common ancestor of the *A. salmonicida* isolates dates back to ~1915 (95% HPD interval 1764 - 1947) and that there have been four main introductions of *A. salmonicida* in Denmark: ~1973 (95% HPD interval 1958 -1979), ~1973 (95% HPD interval 1964 - 1981), ~1948 (95% HPD interval 1934 - 1964) and ~ 1946 (95% HPD interval 1939 - 1961). The four main clades are each shaded with a color SNP differences between major clades are shown above the estimated year of emergence. The three non-Danish *A. salmonicida* each have their own color and have the following labels: Scotland, NCIMB 1102 (type strain 1962), Reference A449 (France 1975). The Danish isolates either have a black color (freshwater farms) or a blue color (seawater farms) and they are labeled by region of origin followed by year of isolation. Following abbreviations are used for regions in Denmark: Nj = Northern Jutland, Mj = Central Jutland, Sd = Southern Denmark, Sj = Zealand. A heatmap illustration with information regarding acquired ARGs, virulence and iron acquisition proteins and plasmid profile numbers of each *A. salmonicida* isolate is shown to the right of the tree. Presence and absence of protein sequences are illustrated by presence and absence of a red square, respectively. Plasmid profile number is shown and isolates that harbor ARGs against multiple antibiotics are labeled with "res". Four minor clades marked with a red ring consist solely of isolates without plasmid pAsa3 and pAsal1.

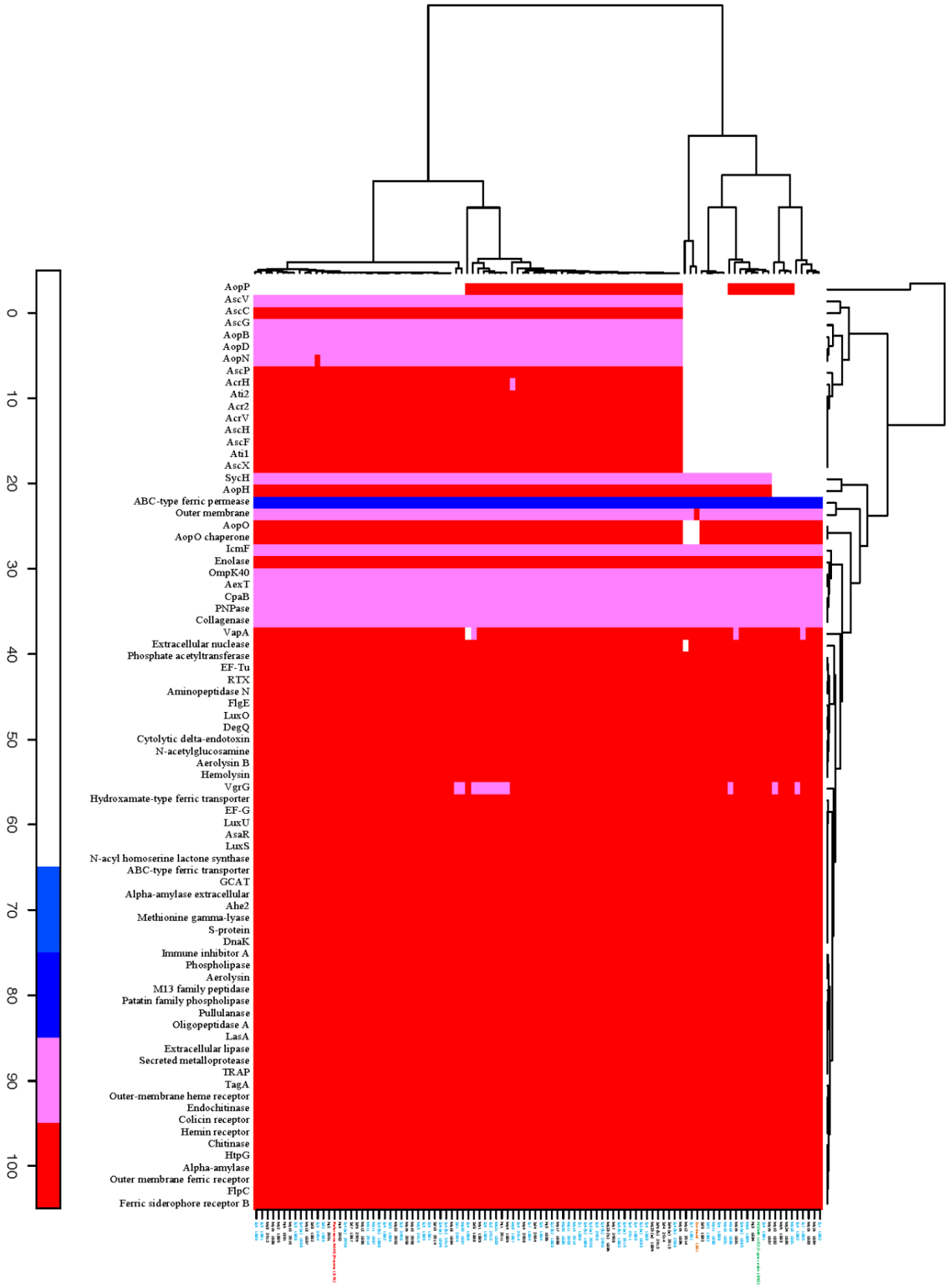
0
10
20
30
40
50
60
70
80
90
100
AopP
AscV
AscC
AscG
AopB
AopD
AopN
AscP
AcrH
Ati2
Acr2
AcrV
AscH
AscF
Ati1
AscX
SycH
AopH
ABC-type ferric permease
Outer membrane
AopO
AopO chaperone
IcmF
Enolase
OmpK40
AexT
CpaB
PNPase
Collagenase
VapA
Extracellular nuclease
Phosphate acetyltransferase
EF-Tu
RTX
Aminopeptidase N
FlgE
LuxO
DegQ
Cytolytic delta-endotoxin
N-acetylglucosamine
Aerolysin B
Hemolysin
VgrG
Hydroxamate-type ferric transporter
EF-G
LuxU
AsaR
LuxS
N-acyl homoserine lactone synthase
ABC-type ferric transporter
GCAT
Alpha-amylase extracellular
Ahe2
Methionine gamma-lyase
S-protein
DnaK
Immune inhibitor A
Phospholipase
Aerolysin
M13 family peptidase
Patatin family phospholipase
Pullulanase
Oligopeptidase A
LasA
Extracellular lipase
Secreted metalloprotease
TRAP
TagA
Outer-membrane heme receptor
Endochitinase
Colicin receptor
Hemin receptor
Chitinase
HtpG
Alpha-amylase
Outer membrane ferric receptor
FlpC
Ferric siderophore receptor B

Figure 2. Heatmap illustrating presence and absence of 78 virulence associated and iron acquisition protein sequences found in the NCBI protein database among the 101 *A. salmonicida* sequenced isolates and the reference A449. Isolates are displayed on the right and sequence protein names on the bottom. Threshold limit for presence of protein in an isolate was set to 75% similarity, expressed as percent sequence identity (ID). Red color represents > 95% ID, pink color > 85% ID, dark blue > 75% ID and light blue > 65% ID.

Supplementary information appendix

Table S1. Full genomic data of the 101 sequenced *A. salmonicida* isolates. Isolates are labeled as stated in Figure 1.

Isolate	Coverage	N50	Contigs	Longest contig	Average contig coverage	Total bps in contigs	Pct. of expected size	Pct. reads in as	Insert Size	Country	Accession no.
Nj2 2001	103	99385	184	381279	304	4696711	94	99	489	Denmark	XXXXXXXX
Mj22 2002	99	110666	201	381769	264	4750938	95	98	493	Denmark	XXXXXXXX
Nj4 2002	128	105305	193	381512	calc. error	4786335	96	98	431	Denmark	XXXXXXXX
Mj11 2003	107	110599	194	381412	486	4697103	94	98	492	Denmark	XXXXXXXX
Sj3 2003	98	115031	207	381370	312	4758893	95	98	500	Denmark	XXXXXXXX
Sd1 2004	91	105305	199	381627	255	4753998	95	99	424	Denmark	XXXXXXXX
Sd4 2004	142	110691	213	381366	calc. error	4759628	95	99	469	Denmark	XXXXXXXX
Sj3 2004	101	102467	202	381541	300	4750816	95	98	484	Denmark	XXXXXXXX
Sd5 2005	86	110641	213	381106	238	4793498	96	99	483	Denmark	XXXXXXXX
Mj7 2008	55	102530	188	381640	193	4690018	94	98	513	Denmark	XXXXXXXX
Mj16 2008	83	110691	159	381687	211	4740131	95	99	469	Denmark	XXXXXXXX
Mj4 2008	108	89390	223	381675	calc. error	4747695	95	98	482	Denmark	XXXXXXXX
Sj3 2008	94	106301	197	381477	249	4751762	95	98	492	Denmark	XXXXXXXX
Sj4 (a) 2009	105	99384	200	381109	290	4750997	95	98	479	Denmark	XXXXXXXX
Sj3 (a) 2009	120	103045	202	381434	calc. error	4758324	95	99	458	Denmark	XXXXXXXX
Sj3 (b) 2009	98	102584	214	381231	288	4757155	95	98	461	Denmark	XXXXXXXX
Sj4 (b) 2009	99	102333	209	381317	313	4756966	95	99	461	Denmark	XXXXXXXX
Mj10 2009	55	99385	216	381398	164	4771942	95	99	449	Denmark	XXXXXXXX
Mj10 2010	88	102393	204	381260	242	4768899	95	98	456	Denmark	XXXXXXXX
Sj3 2010	87	102561	206	381380	calc. error	4758588	95	99	438	Denmark	XXXXXXXX
Sj4 (a) 2010	93	102544	217	381709	309	4757978	95	99	431	Denmark	XXXXXXXX
Sj4 (b) 2010	101	107109	203	381641	calc. error	4753148	95	99	439	Denmark	XXXXXXXX
Nj1 2011	150	110550	228	381259	calc. error	4932368	99	98	463	Denmark	XXXXXXXX
Sj4 2011	98	110673	206	381376	310	4760058	95	99	467	Denmark	XXXXXXXX
Mj9 2012	144	102679	198	381659	402	4753140	95	99	439	Denmark	XXXXXXXX
Mj12 2014	85	110681	166	381442	262	4676044	94	99	463	Denmark	XXXXXXXX
Sd6 (a) 2013	126	110711	203	381684	371	4758827	95	99	451	Denmark	XXXXXXXX
Sd6 (b) 2013	135	102582	209	381428	376	4761737	95	99	459	Denmark	XXXXXXXX
Mj14 2014	78	99257	209	381297	calc. error	4844616	97	97	513	Denmark	XXXXXXXX
Sd4 2014	147	102543	200	381302	457	4755925	95	98	438	Denmark	XXXXXXXX
Mj11 2014	112	102566	209	381692	361	4772818	95	98	427	Denmark	XXXXXXXX
Sd10 2014	117	106303	199	381207	314	4750421	95	99	457	Denmark	XXXXXXXX
Mj3 2014	149	107109	204	381362	411	4769894	95	98	463	Denmark	XXXXXXXX
Sj4 2014	81	110654	212	381386	489	4757435	95	98	513	Denmark	XXXXXXXX
Sj7 1980	29	114971	144	381478	99	4665337	93	98	533	Denmark	XXXXXXXX

Mj24 1980	36	115031	144	381653	177	4641600	93	98	475	Denmark	XXXXXXXX
Mj18 1981	84	110681	166	381396	297	4680471	94	98	531	Denmark	XXXXXXXX
Sj7 1981	130	102554	191	381632	401	4700129	94	99	476	Denmark	XXXXXXXX
Sj4 1981	41	115042	163	381518	194	4661527	93	98	421	Denmark	XXXXXXXX
Mj16 1981	28	96255	170	381112	82	4698702	94	99	448	Denmark	XXXXXXXX
Sd3 1982	31	110578	187	381371	94	4703018	94	99	488	Denmark	XXXXXXXX
Sj4 1982	44	110479	158	381370	129	4669963	93	99	495	Denmark	XXXXXXXX
Sd2 1982	61	110574	181	381663	187	4655791	93	99	460	Denmark	XXXXXXXX
Mj10 1982	35	110592	179	381556	147	4717685	94	98	510	Denmark	XXXXXXXX
Sj4 1983	47	110643	189	381565	185	4745156	95	98	444	Denmark	XXXXXXXX
Mj6 1983	59	110685	171	381612	calc. error	4678164	94	99	422	Denmark	XXXXXXXX
Mj15 1983	52	110673	162	381503	158	4668980	93	99	486	Denmark	XXXXXXXX
Sd2 1983	39	102684	180	381349	126	4740974	95	99	470	Denmark	XXXXXXXX
Sj7 1984	37	102725	189	381428	133	4687108	94	98	433	Denmark	XXXXXXXX
Mj15 1984	43	110650	160	381326	132	4669787	93	99	459	Denmark	XXXXXXXX
Nj3 1984	59	102317	191	381545	208	4702292	94	98	465	Denmark	XXXXXXXX
Mj16 1985	92	102683	186	381167	352	4693921	94	99	449	Denmark	XXXXXXXX
Mj5 1986	51	110600	205	381324	154	4723248	94	99	483	Denmark	XXXXXXXX
Mj18 1986	54	106303	184	381326	201	4704747	94	98	451	Denmark	XXXXXXXX
Mj16 1986	32	99374	208	381361	93	4760365	95	98	478	Denmark	XXXXXXXX
Mj11 1987	58	102503	183	381392	167	4686542	94	99	426	Denmark	XXXXXXXX
Mj16 1987	56	110581	197	381546	170	4723289	94	99	453	Denmark	XXXXXXXX
Mj13 1987	45	110665	204	381556	121	4759024	95	99	488	Denmark	XXXXXXXX
Sj5 1988	85	115073	162	381442	248	4648311	93	99	512	Denmark	XXXXXXXX
Sj4 1988	84	115001	191	381621	336	4698058	94	98	506	Denmark	XXXXXXXX
Sd9 1988	77	102439	236	381519	calc. error	4778057	96	98	478	Denmark	XXXXXXXX
Mj11 1988	112	110588	180	381329	344	4712958	94	99	435	Denmark	XXXXXXXX
Sj1 1989	97	99385	193	381207	403	4696354	94	98	451	Denmark	XXXXXXXX
Sj3 1989	82	118192	147	381415	298	4709626	94	98	497	Denmark	XXXXXXXX
Mj2 1990	105	110638	138	381483	calc. error	4712403	94	99	451	Denmark	XXXXXXXX
Sj3 1990	123	115044	153	381353	392	4708363	94	99	450	Denmark	XXXXXXXX
Mj20 1990	97	99384	206	381475	396	4745926	95	98	488	Denmark	XXXXXXXX
Sj5 1990	147	110649	180	381458	487	4735891	95	99	376	Denmark	XXXXXXXX
Scotland	68	99386	161	381321	234	4672419	93	99	438	Scotland	XXXXXXXX
Sj3 1991	144	110534	187	381129	461	4686788	94	99	407	Denmark	XXXXXXXX
Nj5 1991	145	110468	179	381427	450	4744445	95	99	394	Denmark	XXXXXXXX
Sj5 1991	110	115054	150	381276	368	4708174	94	99	471	Denmark	XXXXXXXX
Mj18 1991	89	110645	170	381628	299	4700329	94	99	453	Denmark	XXXXXXXX
Sj4 1992	109	90171	192	381434	calc. error	4744289	95	99	472	Denmark	XXXXXXXX
Sj1 1992	78	111005	171	381498	248	4700594	94	99	491	Denmark	XXXXXXXX

Sd8 1992	84	102569	187	381652	250	4693600	94	99	523	Denmark	XXXXXXXX
Sj6 (a) 1993	104	110683	184	381352	503	4711783	94	98	481	Denmark	XXXXXXXX
Sj6 (b) 1993	81	102839	210	381188	264	4756685	95	99	420	Denmark	XXXXXXXX
Sj2 (a) 1993	173	102788	201	381562	564	4757469	95	99	367	Denmark	XXXXXXXX
Sj2 (b) 1993	119	102537	209	381325	348	4756068	95	99	443	Denmark	XXXXXXXX
Mj21 1993	107	105305	200	381727	323	4760383	95	99	478	Denmark	XXXXXXXX
Mj19 1994	77	105305	187	381317	206	4693576	94	99	482	Denmark	XXXXXXXX
Sj5 1994	49	99385	207	381506	146	4757079	95	98	521	Denmark	XXXXXXXX
Nj3 1995	90	102828	202	381519	263	4758748	95	98	465	Denmark	XXXXXXXX
Mj12 1995	133	110503	167	381263	431	4737917	95	99	408	Denmark	XXXXXXXX
Mj1 1995	126	110655	170	381310	calc. error	4716005	94	99	373	Denmark	XXXXXXXX
Mj4 1995	117	102745	197	381303	423	4746620	95	98	431	Denmark	XXXXXXXX
Sj5 1995	89	102519	200	381605	274	4758268	95	98	472	Denmark	XXXXXXXX
Sd2 1995	129	102730	205	381625	395	4754891	95	99	430	Denmark	XXXXXXXX
Mj23 (a) 1996	121	102588	175	381681	388	4737728	95	98	466	Denmark	XXXXXXXX
Mj23 (b) 1996	124	110588	166	381327	388	4688488	94	99	508	Denmark	XXXXXXXX
Sj6 (a) 1996	62	110688	202	381738	calc. error	4755441	95	98	560	Denmark	XXXXXXXX
Sj6 (b) 1996	101	105305	197	381146	calc. error	4697453	94	99	430	Denmark	XXXXXXXX
Mj8 1997	164	99757	208	381552	598	4779438	96	99	404	Denmark	XXXXXXXX
Sd7 1997	59	102690	182	381336	164	4741454	95	99	503	Denmark	XXXXXXXX
Mj20 1997	108	102547	205	381430	calc. error	4749870	95	99	374	Denmark	XXXXXXXX
Sj4 1998	77	110635	187	381495	387	4697323	94	98	480	Denmark	XXXXXXXX
Sj4 (a) 1999	72	110581	160	484118	193	4735436	95	99	485	Denmark	XXXXXXXX
Mj17 1999	93	110527	186	381251	calc. error	4697920	94	98	516	Denmark	XXXXXXXX
Sj4 (b) 1999	86	99385	197	381581	228	4707087	94	99	496	Denmark	XXXXXXXX
NCIMB 1102 (Type strain 1962)	91	103414	195	381570	552	4713735	94	97	482	England	XXXXXXXX

Table S2. Overview of 78 virulence associated and iron acquisition protein sequences found in the NCBI protein database. The following is shown in the table: name of protein sequence, short description, location of the protein coding gene, Genbank accession number and the number of *A. salmonicida* isolates harboring the given protein sequence. Isolates are labeled as stated in Figure 1.

Protein	Short description of protein function	Gene location	Genbank accession no.	Present in no. of isolates
AopP	T3SS effector protein	pAsal1	YP_009062872.1	51
AscV	T3SS inner membrane export apparatus	pAsa5	EHI50383.1	77
AscC	T3SS protein of the outer membrane ring	pAsa5	ABD48952.1	77
AscG	T3SS chaperone	pAsa5	ABO92527.1	77
AscP	T3SS needle length control; ruler protein; regulation of secretion; substrate specifity switch	pAsa5	EHI50374.1	77
AscH	T3SS regulator needle assembling	pAsa5	EHI50401.1	77
AscF	T3SS early substrate, needle subunit	pAsa5	EHI50399.1	77
AscX	T3SS translocator needle subunit chaperoned by AscY	pAsa5	ABO92546.1	77
AcrH	T3SS chaperone for AopB/AopD	pAsa5	EHI50387.1	77
Acr2	T3SS chaperone	pAsa5	ABO92547.1	77
AcrV	T3SS middle substrate; tip translocon; hydrophilic translocators; protective antigen; anti-host factor	pAsa5	ABO92541.1	77
AopB	T3SS translocon; hydrophobic translocators; pore in host cell	pAsa5	EHI50388.1	77
AopD	T3SS translocon; hydrophobic translocators; pore in host cell	pAsa5	EHI50389.1	77
AopN	T3SS secretion control of translocators and immune suppressor	pAsa5	EHI50378.1	77
Ati2	T3SS inositol polyphosphate 5-phosphatase	pAsa5	ABO92519.1	77
Ati1	T3SS translocator needle subunit (Ati2 chaperone)	pAsa5	ABO92520.1	77
AopH	T3SS putative tyrosine phosphatase	pAsa5	ABD48950.1	93
SycH	T3SS AopH chaperone	pAsa5	ABO92484.1	93
AopO	T3SS putative serine/threonine kinase	pAsa5	ABD48951.1	99
AopO chaperone	T3SS AopO chaperone	pAsa5	ABO92569.1	99
VapA (A-layer protein)	Tetragonal virulence array protein; coats surface of bacteria; confers virulence to bacteria	Chromosome	EHI51039.1	101
Extracellular nuclease	Secreted enzyme; cell communication	Chromosome	ABO90268.1	101
AexT	T3SS secreted toxin; ADP-ribosyltransferase	Chromosome	ABD48949.1	102
FlpC	Type IV pilus secretin	Chromosome	ABD57354.1	102
IcmF	T6SS protein	Chromosome	KFN20003.1	102
VgrG	T6SS effector protein	pAsa4 (homolog on chromosome)	KIX26565.1	102
S-protein	T2SS S-protein secretion component E	Chromosome	ABO89526.1	102
Aerolysin	Aerolysin toxin	Chromosome	ABO91859.1	102
Aerolysin B	Aerolysin B toxin	Chromosome	ABO90867.1	102
EF-G	Elongation factor G	Chromosome	A4SHV8.2	102

EF-Tu	Elongation factor Tu	Chromosome	A4SHU2.1	102
DnaK	Molecular chaperone	Chromosome	EHI53225.1	102
HtpG	Heat shock protein; ATPase activity	Chromosome	ABO89902.1	102
PNPase	mRNA degradation; 3'-5'-exoribonuclease activity; magnesium ion binding	Chromosome	A4SJR9.1	102
Aminopeptidase N	Aminopeptidase activity; metallopeptidase activity; zinc ion binding	Chromosome	EHI52287.1	102
Methionine gamma-lyase	Lyase activity; pyridoxal phosphate binding	Chromosome	ABO90396.1	102
Phosphate acetyltransferase	Phosphate acetyltransferase activity; acetyl-CoA biosynthetic process	Chromosome	ABO91377.1	102
Extracellular lipase	Triglyceride lipase activity	Chromosome	EHI50452.1	102
TagA	ToxR-regulated lipoprotein; metalloendopeptidase activity	Chromosome	EHI53531.1	102
Ahe2	Serine-type endopeptidase activity	Chromosome	EHI54143.1	102
N-acetylglucosamine	N-acetylglucosamine-binding protein A; viral capsid	Chromosome	KHF01495.1	102
Enolase	Magnesium ion binding; phosphopyruvate hydratase activity; glycolytic process	Chromosome	EHI51131.1	102
Outer membrane	Outer membrane protein	Chromosome	ABO89374.1	102
OmpK40	Outer membrane porin II	Chromosome	EHI50654.1	102
DegQ	Serine-type endopeptidase activity	Chromosome	EHI52649.1	102
Hemolysin	Hemolysis in other organism; toxin	Chromosome	EHI53183.1	102
Alpha-amylase extracellular	Alpha-amylase activity; cation binding; carbohydrate metabolic process	Chromosome	ABO89392.1	102
Alpha-amylase	Alpha-amylase activity; cation binding; carbohydrate metabolic process	Chromosome	ABO91424.1	102
Secreted metalloprotease	Metallopeptidase activity	Chromosome	EHI52510.1	102
collagenase	Serine-type endopeptidase activity; zinc ion binding	Chromosome	EHI52177.1	102
GCAT	Lipase activity; transferase activity, transferring acyl groups	Chromosome	ABO88676.1	102
Chitinase	Carbohydrate binding ; chitinase activity	Chromosome	ABO90207.1	102
Endochitinase	Carbohydrate binding ; chitinase activity	Chromosome	ABO91302.1	102
Pullulanase	Carbohydrate binding; pullulanase activity	Chromosome	EHI52980.1	102
RTX	Calcium ion binding	Chromosome	ABO88975.1	102
Cytolytic delta-endotoxin	Insecticidal protein; pathogenesis	Chromosome	EHI53783.1	102
Patatin family phospholipase	Lipid metabolic process	Chromosome	EHI53765.1	102
Phospholipase	phospholipase C precursor	Chromosome	EHI52973.1	102
ABC-type ferric transporter	ABC-type ferric siderophore transporter; periplasmic binding protein	Chromosome	ABO92284.1	102
Ferric siderophore receptor B	Iron ion binding; receptor activity; siderophore transport	Chromosome	ABO92283.1	102
Hydroxamate-type ferric transporter	ABC-type hydroxamate-type ferric siderophore transporter; periplasmic binding protein	Chromosome	ABO92281.1	102
Outer membrane ferric receptor	Iron ion binding; receptor activity; siderophore transport	Chromosome	ABO91837.1	102
ABC-type ferric permease	Transporter activity	Chromosome	ABO92286.1	102
Outer-membrane heme receptor	Receptor activity; transporter activity	Chromosome	ABO91308.1	102
Hemin receptor	Receptor activity; transporter activity	Chromosome	ABO90714.1	102

Colicin receptor	Receptor activity; transport	Chromosome	ABO89926.1	102
N-acyl homoserine lactone synthase	N-acyl homoserine lactone synthaseactivity, quorum sensing	Chromosome	EHI52135.1	102
LuxS	Iron ion binding; S-ribosylhomocysteine lyase activity; quorum sensing	Chromosome	A4SIY8.1	102
AsaR	Transcriptional activator; DNA binding	Chromosome	ABO91724.1	102
LuxU	Phosphorelay protein; signal transducer activity	Chromosome	ABO90799.1	102
LuxO	Phosphorelay signal transduction system; ATP binding; sequence-specific DNA binding	Chromosome	ABO91277.1	102
CpaB	Flp pilus assembly protein	Chromosome	EHI53195.1	102
FlgE	flagellar hook protein	Chromosome	EHI52692.1	102
Oligopeptidase A	Metal ion binding; metalloendopeptidase activity	Chromosome	ABO92225.1	102
M13 family peptidase	Metalloendopeptidase activity	Chromosome	EHI53386.1	102
TRAP	Transporter solute receptor; TAXI family	Chromosome	ABO91932.1	102
LasA	Metalloendopeptidase activity	Chromosome	ABO89393.1	102
Immune inhibitor A	Metalloendopeptidase activity	Chromosome	ABO88993.1	102

Table S3. Overview of 11 *A. salmonicida* plasmids found in the NCBI nucleotide database. The following is shown in the table: name of plasmid, short description, antibiotic resistance genes encoded on plasmid, length of plasmid in base pairs (bp), and Genbank accession number. Isolates are labeled as stated in Figure 1.

Plasmid	Short description	Antibiotic resistance genes	Length (bp)	Genbank accession no.
pAsa1	Small cryptic plasmid		5424	NC_004923.1
pAsa2	Small cryptic plasmid		5247	NC_004925.1
pAsa3	Small cryptic plasmid		5616	NC_004924.1
pAsa4	Large plasmid encoding three T6SS proteins	*aadA1, cat, sul1, tet(E)*	166749	NC_009349.1
pAsa5	Large plasmid encoding the majority of T3SS proteins		155098	NC_009350.1
pAsa6	Medium plasmid encoding homologs of the T3SS Effector protein AopH and AopH chaperone		18536	NC_009352.2
pAsal1	Small plasmid encoding the T3SS Effector protein AopP		6371	NC_004338.1
pAr_32	Sequence from the pAr-32 resistance-determining region	*aadA2, catA2, sul1*	9340	AJ517791.1
pRAS1	Sequence from the pRAS1 resistance-determining region	*sul1, tet(A), dfrA16*	11663	AJ517790.2
pRAS3.1	Non-conjugative Tet C plasmid	*tet(C)*	11851	NC_003123.1
pRAS3.2	Non-conjugative Tet C plasmid (variant of pRAS3.1)	*tet(C)*	11823	NC_003124.1

Table S4. Plasmid content of the 101 *A. salmonicida* sequenced isolates and the reference A449. Numbers under each plasmid name represent percent coverage of that plasmid (in base pairs) for a given isolate. If percent coverage is higher than 60%, the plasmid is said to be present in the isolate and the color of the cell is green. Threshold limit for presence of plasmid in an isolate was set to 75% similarity expressed as percent sequence identity (ID) and 60% of alignment length (coverage) of the plasmid. Following abbreviations are used for percent coverage: 100 = 100% coverage, <100 = from 80% up to 99% coverage, <80 = from 60% up to 79% coverage, <60 = from 10 up to 59% coverage, <10 = from 1% up to 9% coverage, 0 = 0% coverage. Isolates are labeled as stated in Figure 1.

A. salmonicida isolate	pAsa1	pAsa2	pAsa3	pAsa4	pAsa5	pAsa6	pAsal1	pAr-32	pRAS1	pRAS3.1	pRAS3.2	Plasmid profile no.
Sd4 2004	100	< 80	100	< 10	< 100	< 80	100	0	0	0	0	1
Mj7 2008	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Nj1 2011	100	< 100	< 100	< 60	< 100	< 80	100	0	0	0	0	1
Sd6 (a) 2013	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sd6 (b) 2013	100	100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sd4 2014	100	< 80	< 100	< 10	< 100	< 80	100	0	0	0	0	1
Mj16 1981	100	< 100	< 100	< 10	< 80	< 80	100	0	< 10	0	0	1
Nj3 1984	100	< 100	100	< 10	< 80	< 80	100	0	< 10	0	0	1
Mj16 1987	100	< 100	< 100	< 10	< 80	< 80	100	0	0	0	0	1
Sd9 1988	100	< 100	100	< 10	100	< 80	100	0	0	0	0	1
Nj3 1995	100	< 80	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj1 1995	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj4 1995	100	< 100	100	< 10	< 100	< 80	100	0	0	0	0	1
Mj23 (a) 1996	100	< 100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj23 (b) 1996	100	< 80	< 100	< 10	< 100	< 80	100	0	0	0	0	1
Mj17 1999	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj11 2003	100	< 100	100	0	< 100	< 80	100	0	< 10	0	0	1
Sj3 2003	100	< 100	100	< 10	< 100	< 80	100	0	0	0	0	1
Sj3 (a) 2009	100	< 100	< 100	< 10	< 100	< 80	100	0	0	0	0	1
Sj3 (b) 2009	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj4 (b) 2009	100	100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj3 2010	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj4 (a) 2010	100	< 80	100	< 10	< 100	< 80	100	0	0	0	0	1
Sj4 2011	100	100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj14 2014	100	< 100	100	< 60	< 100	< 80	100	0	0	0	0	1

Sj4 2014	100	< 80	100	< 10	< 100	< 80	100	0	0	0	0	1
Sj4 1981	100	< 100	100	< 10	< 80	< 80	100	0	0	0	0	1
Sj4 1983	100	< 80	100	< 10	< 100	< 80	100	0	0	0	0	1
Sj7 1984	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj5 1986	100	< 100	100	< 10	< 80	< 80	100	0	0	0	0	1
Mj18 1986	100	< 100	100	< 10	< 80	< 80	100	0	0	0	0	1
Sj4 1988	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj1 1989	100	< 100	100	< 10	< 100	< 80	100	0	0	0	0	1
Mj20 1990	100	< 100	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj6 (a) 1993	100	< 100	100	< 10	< 80	< 80	100	0	0	0	0	1
Sj6 (b) 1993	100	< 80	< 100	< 10	< 100	< 80	100	0	0	0	0	1
Sj2 (a) 1993	100	< 100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj2 (b) 1993	100	100	< 80	< 10	< 100	< 80	< 100	0	< 10	0	0	1
Mj21 1993	100	< 100	100	< 10	< 100	< 80	100	0	0	0	0	1
Sj5 1994	100	100	< 100	< 10	< 100	< 80	100	0	< 10	0	0	1
Sj5 1995	100	< 80	< 100	< 10	< 100	< 80	100	0	0	0	0	1
Sj6 (b) 1996	100	< 80	100	< 10	< 100	< 80	100	0	< 10	0	0	1
Mj8 1997	100	< 100	100	< 10	< 100	< 80	100	0	< 10	< 60	< 60	1
Sj4 1998	100	< 100	100	< 10	< 100	< 80	100	0	0	0	0	1
NCIMB 1102 (Type strain 1962)	100	< 100	100	< 10	< 80	< 80	100	0	0	0	0	1
Mj4 2008	100	< 10	100	< 10	< 100	< 80	< 100	0	0	0	0	2
Sj7 1981	100	< 100	< 100	< 10	< 60	< 80	100	0	0	0	0	3
Scotland	100	< 100	< 100	< 10	< 60	< 80	100	0	0	0	0	3
Mj24 1980	100	< 80	100	< 10	< 60	< 60	100	0	0	0	0	4
Mj10 1982	100	< 100	100	< 10	< 60	< 60	100	< 60	< 60	0	0	4
Mj6 1983	100	< 100	100	< 10	< 60	< 60	100	0	0	0	0	4
Mj18 1981	100	< 80	100	< 10	< 60	< 60	100	0	0	0	0	4
Mj16 1986	100	< 80	< 100	< 10	< 100	< 60	100	0	0	0	0	5
Mj15 1983	100	< 100	100	< 10	< 60	< 60	< 60	0	0	0	0	6
Mj15 1984	100	< 100	100	< 10	< 60	< 60	< 60	0	0	0	0	6
Sj4 1982	100	< 80	100	< 10	< 60	< 60	< 60	0	0	0	0	6
Mj12 2014	100	< 80	100	< 10	< 60	< 80	< 60	0	< 10	0	0	7
Sj4 (a) 2009	< 100	100	< 80	< 10	< 100	< 60	< 60	0	0	0	0	8
Sj7 1980	100	< 100	100	< 10	< 80	< 60	< 60	0	0	0	0	8

Sj4 1992	100	< 100	100	< 10	< 100	< 60	< 60	0	0	0	0	8
Sj1 1992	100	< 80	100	< 10	< 80	< 60	< 60	0	0	0	0	8
Sj4 (b) 2010	< 100	< 80	< 60	< 10	< 100	< 60	< 60	0	< 10	0	0	9
Sj4 (a) 1999	< 100	< 100	0	< 10	< 80	< 60	< 60	0	0	0	0	9
Mj22 2002	< 100	< 100	< 60	< 10	< 100	< 80	< 60	0	0	0	0	10
Nj4 2002	< 100	< 100	< 60	< 10	100	< 80	< 60	0	< 10	0	0	10
Sd5 2005	< 100	< 80	< 10	< 10	< 100	< 80	< 60	< 60	< 60	0	0	10
Mj16 2008	< 100	< 100	0	< 10	100	< 100	< 60	0	0	0	0	10
Mj10 2009	< 100	< 100	< 10	< 10	< 100	< 80	< 60	< 10	0	< 60	< 60	10
Mj10 2010	< 100	100	0	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Mj9 2012	< 80	< 100	0	< 10	< 80	< 80	< 60	0	0	0	0	10
Sd10 2014	< 100	100	< 10	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Mj13 1987	< 100	< 100	< 10	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Mj2 1990	< 100	< 80	0	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Mj12 1995	< 80	< 100	< 60	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Sd1 2004	< 80	< 100	0	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Sj3 2004	< 100	< 100	< 60	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Sj3 2008	< 80	100	< 60	< 10	< 100	< 80	< 60	0	0	0	0	10
Mj11 2014	< 80	< 100	0	< 10	< 100	< 80	< 60	0	0	6	6	10
Mj3 2014	< 100	< 100	< 10	< 10	< 100	< 80	< 60	< 60	< 60	0	0	10
Sj5 1988	< 100	< 80	< 60	< 10	< 80	< 80	< 60	0	0	0	0	10
Mj18 1991	< 100	< 80	< 60	< 10	< 80	< 80	< 60	0	0	0	0	10
Sd2 1995	< 100	< 80	0	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Sj6 (a) 1996	< 100	< 100	< 60	< 10	< 100	< 80	< 60	0	0	0	0	10
Mj20 1997	< 80	< 100	< 60	< 10	< 100	< 80	< 60	0	< 10	0	0	10
Sj4 (b) 1999	< 100	< 100	0	< 10	< 100	< 80	< 60	0	0	0	0	10
Nj2 2001	100	< 100	100	< 10	< 100	< 80	< 60	0	< 10	0	0	11
Sd3 1982	100	< 80	100	< 10	< 80	< 80	< 60	0	< 10	0	0	11
Mj16 1985	100	< 100	100	< 10	< 100	< 80	< 60	0	< 10	0	0	11
Nj5 1991	100	< 100	100	< 10	< 100	< 80	< 60	0	< 10	0	0	11
Sd8 1992	100	< 100	100	< 10	< 100	< 80	< 60	0	0	0	0	11
Mj19 1994	100	< 100	100	< 10	< 100	< 80	< 60	0	< 10	0	0	11
Sd7 1997	100	< 100	100	< 10	< 100	< 80	< 60	0	< 10	0	0	11
Sd2 1982	100	< 80	100	< 10	< 80	< 80	< 60	0	0	0	0	11

Sd2 1983	**100**	**< 100**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**< 10**	**0**	**0**	**11**
Mj11 1987	**100**	**< 80**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**< 10**	**0**	**0**	**11**
Mj11 1988	**100**	**< 100**	**100**	**< 10**	**< 80**	**< 80**	**< 60**	**0**	**0**	**0**	**0**	**11**
Sj3 1989	**100**	**< 80**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**< 10**	**0**	**0**	**11**
Sj3 1990	**100**	**< 80**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**< 10**	**0**	**0**	**11**
Sj5 1990	**100**	**< 100**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**0**	**0**	**0**	**11**
Sj3 1991	**100**	**< 100**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**0**	**0**	**0**	**11**
Sj5 1991	**100**	**< 100**	**100**	**< 10**	**< 100**	**< 80**	**< 60**	**0**	**0**	**0**	**0**	**11**
Reference A449 (France 1975)	**< 100**	**< 100**	**100**	**100**	**< 100**	**0**	**0**	**0**	**0**	**0**	**0**	**12**
TOTAL	**102**	**101**	**78**	**1**	**92**	**87**	**53**	**0**	**0**	**0**	**0**	**12**

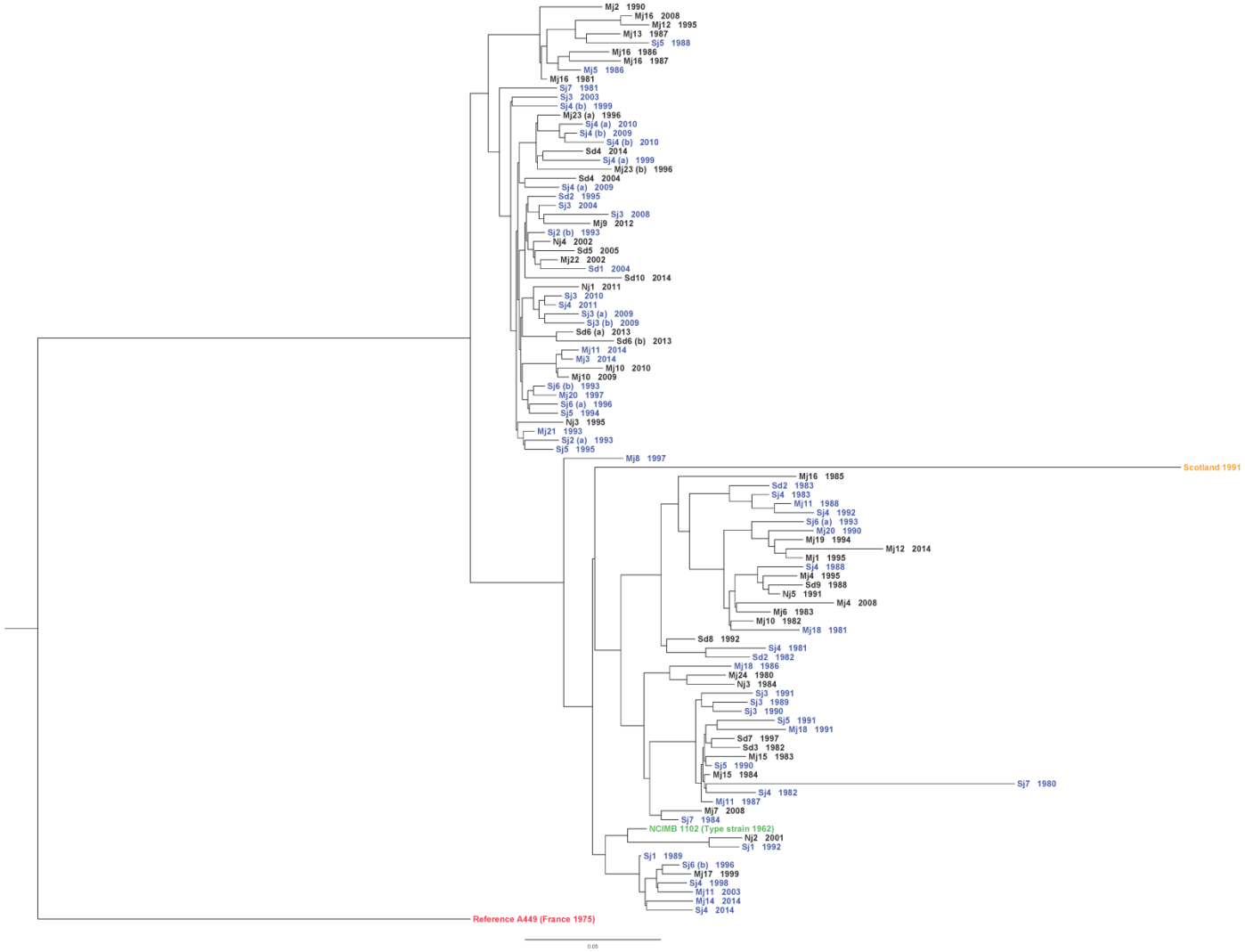

Figure S1. Phylogeny of *A. salmonicida.* Maximum likelihood tree based on the alignment of 667 SNPs found among the 101 *A. salmonicida* sequenced isolates and the reference A449. Isolates are labeled as stated in Figure 1.